(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
15 March 2001 (15.03.2001)

PCT

(10) International Publication Number
WO 01/18044 A3

(51) International Patent Classification[7]: C07K 14/47, 14/705, A61K 38/17, A61P 35/00, 37/02

(21) International Application Number: PCT/US00/24560

(22) International Filing Date:
7 September 2000 (07.09.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

60/152,914	8 September 1999 (08.09.1999)	US
60/156,257	27 September 1999 (27.09.1999)	US
60/173,906	29 December 1999 (29.12.1999)	US

(63) Related by continuation (CON) or continuation-in-part (CIP) to earlier applications:

US	60/152.914 (CON)
Filed on	8 September 1999 (08.09.1999)
US	60/156,257 (CON)
Filed on	27 September 1999 (27.09.1999)
US	60/173,906 (CON)
Filed on	29 December 1999 (29.12.1999)

(71) Applicant *(for all designated States except US)*: **IMMUNEX CORPORATION** [US/US]; 51 University Street, Seattle, WA 98101-2936 (US).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: **SPRIGGS, Melanie, K.** [US/US]; 2256 - 12th Avenue West, Seattle, WA 98119 (US).

(74) Agent: **DAVIES, Tracey, B.**; Vinson & Elkins L.L.P., 2300 First City Tower, 1001 Fannin, Houston, TX 77002-6760 (US).

(81) Designated States *(national)*: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

(88) Date of publication of the international search report:
25 October 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/18044 A3

(54) Title: USE OF SEMAPHORIN FOR MODULATION OF CELLULAR EFFLUX

(57) Abstract: The present invention is directed to compositions and methods comprising the use of semaphorins, semaphorin receptors, and polynucleotides encoding semaphorins and semaphorin receptors, for the modulation of cellular efflux mumps. Included in the present invention are compositions and methods for control of multiple drug resistance phenotypes *via* control of cellular efflux.

INTERNATIONAL SEARCH REPORT

Inte. .onal Application No
PCT/US 00/24560

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/47 C07K14/705 A61K38/17 A61P35/00 A61P37/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, CHEM ABS Data, EMBASE, MEDLINE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 99 45114 A (ZYMOGENETICS) 10 September 1999 (1999-09-10) page 51 claims 1-37 ---	1
A	TESSHI YAMADA ET AL: "Identification of semaphorin E as a non-MDR drug resistance gene of human cancers" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCE USA, vol. 94, December 1997 (1997-12), pages 14713-14718, XP002164646 cited in the application page 14713, rignt-hand column, line 1 - line 2 page 14717 --- -/--	1

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

'A' document defining the general state of the art which is not considered to be of particular relevance

'E' earlier document but published on or after the international filing date

'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

'O' document referring to an oral disclosure, use, exhibition or other means

'P' document published prior to the international filing date but later than the priority date claimed

'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

'&' document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 April 2001	25/04/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Siatou, E

2

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inte. onal Application No
PCT/US 00/24560

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	J. H. WEISBURG ET AL: "Intracellular pH and Multidrug Resistance Regulate Complement-mediated Cytotoxicity of Nucleated Human Cells " THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 274, no. 16, 16 April 1999 (1999-04-16), pages 10877-10888, XP002164647 cited in the application abstract	1
A	ALTAN ET AL: "Defective acidification in human breast tumor cells and implications for chemotherapy" JOURNAL OF EXPERIMENTAL MEDICINE,JP,TOKYO, vol. 187, no. 10, 18 May 1998 (1998-05-18), pages 1583-1598, XP002118974 ISSN: 0022-1007 cited in the application abstract	1
A	M SCHINDLER ET AL: "Defective pH Regulation of Acidic Compartments in Human Breast Cancer Cells (MCF-7) Is Normalized in Adriamycin-Resistant Cells (MCF-7adr)" BIOCHEMISTRY,US,AMERICAN CHEMICAL SOCIETY. EASTON, PA, vol. 35, no. 9, 5 March 1996 (1996-03-05), pages 2811-2817, XP002118976 ISSN: 0006-2960 cited in the application abstract	1
A	WO 99 21997 A (IMMUNEX CO) 6 May 1999 (1999-05-06) claims 1-19	1
A	WO 95 07706 A (UNIVERSITY OF CALIFORNIA) 23 March 1995 (1995-03-23) the whole document & US 5 935 865 A 10 August 1999 (1999-08-10) cited in the application	1

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte. onal Application No
PCT/US 00/24560

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9945114 A	10-09-1999	AU 2982799 A	20-09-1999
WO 9921997 A	06-05-1999	AU 1204799 A	17-05-1999
		EP 1027436 A	16-08-2000
		US 6187909 B	13-02-2001
WO 9507706 A	23-03-1995	US 5639856 A	17-06-1997
		AU 683494 B	13-11-1997
		AU 7724094 A	03-04-1995
		CA 2171638 A	23-03-1995
		EP 0721342 A	17-07-1996
		JP 9505725 T	10-06-1997
		US 5935865 A	10-08-1999
		US 6013781 A	11-01-2000
		US 5807826 A	15-09-1998

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
15 March 2001 (15.03.2001)

PCT

(10) International Publication Number
WO 01/18044 A2

(51) International Patent Classification[7]: C07K 14/00

(21) International Application Number: PCT/US00/24560

(22) International Filing Date:
7 September 2000 (07.09.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

60/152,914	8 September 1999 (08.09.1999)	US
60/156,257	27 September 1999 (27.09.1999)	US
60/173,906	29 December 1999 (29.12.1999)	US

(63) Related by continuation (CON) or continuation-in-part (CIP) to earlier applications:

US	60/152.914 (CON)
Filed on	8 September 1999 (08.09.1999)
US	60/156,257 (CON)
Filed on	27 September 1999 (27.09.1999)
US	60/173,906 (CON)
Filed on	29 December 1999 (29.12.1999)

(71) Applicant *(for all designated States except US)*: **IMMUNEX CORPORATION** [US/US]; 51 University Street, Seattle, WA 98101-2936 (US).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: **SPRIGGS, Melanie, K.** [US/US]; 2256 - 12th Avenue West, Seattle, WA 98119 (US).

(74) Agent: **DAVIES, Tracey, B.**; Vinson & Elkins L.L.P., 2300 First City Tower, 1001 Fannin, Houston, TX 77002-6760 (US).

(81) Designated States *(national)*: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

— *Without international search report and to be republished upon receipt of that report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/18044 A2

(54) Title: SEMAPHORIN MODULATION OF CELLULAR EFFLUX

(57) Abstract: The present invention is directed to compositions and methods comprising the use of semaphorins, semaphorin receptors, and polynucleotides encoding semaphorins and semaphorin receptors, for the modulation of cellular efflux mumps. Included in the present invention are compositions and methods for control of multiple drug resistance phenotypes *via* control of cellular efflux.

SEMAPHORIN MODULATION OF CELLULAR EFFLUX

This application claims priority to United States provisional patent application serial number 60/152,914 filed September 8, 1999, now abandoned; United States provisional patent application serial number 60/156,257, filed September 27, 1999, now abandoned; and United States provisional patent application serial number 60/173,906 filed December 29, 1999, now abandoned.

I. FIELD OF THE INVENTION

The present invention relates to compositions and methods useful in manipulating cellular efflux mechanisms resulting in multiple drug resistance (MDR). More specifically, the present invention relates to the use of semaphorin or semaphorin receptor polypeptides, as well as polynucleotides encoding these polypeptides, to modulate cellular efflux or the MDR phenotype of cells.

II. BACKGROUND OF THE INVENTION

In response to unavoidable continuous exposure to a frequently hostile environment, cells have developed a multitude of mechanisms to prevent entry or to accelerate exit of noxious substances from the intra-cellular space. This "cellular Darwinism" is accepted as a basic tool of survival, but, once applied by targeted cells to cytotoxic drugs, the phenomenon interferes with the effectiveness of chemotherapies for an array of diseases such as cancer and HIV. As the result of a wide spectrum of highly effective systems, drug resistance, whatever its source, is a prevalent cause for chemotherapeutic failure.

When cellular resistance to one drug results in resistance to a wide array of chemical agents, including those that are not related to the substance originally inducing the resistance, the cell is regarded as having developed multidrug resistance, or MDR. Thus, MDR is a cellular phenomenon characterized by resistance of the cell to cytotoxic substances. Generally, MDR develops in response to a specific cytotoxic substance, but then confers resistance to an array of cytotoxic substances or conditions. Cells that have developed MDR are considered MDR phenotypic cells, and are further described as those cells that have an increased ability, relative to non-MDR cells, to survive in the presence of cytotoxic substances or cytotoxic conditions. The increased survival rates of MDR phenotypic cells is characteristically due to an increased cellular capacity to efflux or expel from the cell substances that are either cytotoxic in themselves, or are present in the cell in cytotoxic amounts, thereby creating a cytotoxic condition for the cell. In an attempt to understand and control MDR, many investigators have studied the various mechanisms thought to drive it. *See* Kellen, Alternative Mechanisms of Multidrug Resistance in Cancer, (1995). MDR phenotypes of cancer or other cells may arise as a result of MDR proteins, or MDR-like proteins, or various other mechanisms involving efflux pumps. Cellular efflux pumps involved in the development of MDR phenotypic cells include those that are able to efflux molecules of many different sizes and compositions, as well as protons or

chloride ions. For example, MDR protein pumps include the proteins MDR-1 and MDR-2, which are each considered to be a *P-glycoprotein* (*P-gp*), and the human multiple drug resistance associated protein designated "MRP" (*see*, Zaman, *et al.*, 1994). These and other MDR proteins are transmembrane efflux pumps that, based on studies in the mouse, are believed to be important in removing toxins from the cell.

Various assays that have been developed to allow the study of exchange of molecules across membranes are employed in the study of MDR proteins. For example, many lipophilic, cationic dyes have been described that allow one to follow changes in membrane potential, or changes in intracellular pH. One such dye, Rhodamine 123 (Rh123) was frequently used by hematologists to measure mitochondrial membrane potential, and has been described as a substrate for MDR proteins. Kim *et.al.*, (1998). Consistent with the reported transport of protons, expression of the MDR protein *P-gp* has been associated with a significant elevation of intracellular pH (Weisburg *et al*, 1999).

Further, MDR phenotypes are reported to arise in some cell types as a result of alterations in the acidification (pH) of intracellular organelles and compartments, such as the trans-golgi network and the endocytic pathway (*see, e.g.*, Altan, *et al.*, Altan, N *et al.*, Chen, Y *et al.*, Schindler, *et al.*). One mechanism for controlling the pH of intracellular compartments is by cellular pumps that operate to move protons, or negatively charged ions like chloride ions, across membranes. Such cellular pumps are implicated in certain diseases. For example, unregulated activity of a chloride pump is known to be at least partially responsible for the development of cystic fibrosis resulting from a genetic defect. Alternatively, growth factors are theorized to play a non-efflux-related role in MDR. For example, semaphorins have been postulated to function as growth factors, and thereby exert an effect on cells that may contribute to the development of drug resistance (Yamada, *et al.*).

In light of the various relationships between cellular efflux pumps and MDR, the ability to control such efflux pumps would provide the ability to promote or suppress the development of MDR in cells. Accordingly, investigation into MDR mechanisms, and various methods for controlling MDR *via* control of cellular efflux mechanisms is ongoing.

III. SUMMARY OF THE INVENTION

The present invention teaches the use of semaphorin or semaphorin receptor polypeptides to modulate the activity of cellular efflux pumps. The present invention further teaches that semaphorin or semaphorin receptor polypeptides can be used to specifically activate or inhibit cellular efflux pumps and therefore may induce or inhibit the development of multiple drug resistant cells. The present invention further provides compositions and methods for the treatment of neoplasms, autoimmune or immuno-deficiency disorders such as HIV, and other cellular-efflux-related disease states.

The present invention specifically contemplates that any semaphorin polypeptide, or active fragment of a semaphorin polypeptide, may be used in the disclosed compositions and methods. Exemplary semaphorins include, for example and without limitation: AHV Sema; A39R; Sema I, including G-sema I and D-sema-I; Sema II; Sema III; Sema IV; DC Sema; CD100; Z SMF-7; Sema A; Sema B; Sema C; Sema D; Sema E; Sema H; Sema L; Sema W and Sema Y. Additionally, useful fragments of any semaphorin, such as the sema domain or the active domain may also be used according to the present invention. For additional semaphorins that can be used in the presently disclosed compositions and methods, *see* Bamberg, *et.al.* Cell, 97:551 and United States Patent No. 5,935,865 to Goodman *et al.* In alternative embodiments of the present invention, such as in "gene therapeutics," nucleic acid sequences encoding any of these semaphorins or their fragments can be used.

Similarly, preferred semaphorin receptor polypeptides for use in the presently disclosed compositions and methods include those semaphorin receptors known as plexins, as well as their complements, variants and useful fragments such as soluble portions of the receptors, fragments including the sema domain of the plexins, and fragments including the active sites of the plexins. A particularly preferred plexin for use according to the present invention is the Viral-Encoded Semaphorin Receptor ("VESPR"), as well as complements, variants, and soluble fragments thereof. Particularly preferred polypeptide sequences include the polypeptide sequence of SEQ ID NO:2. Additionally, useful soluble forms of the VESPR polypeptide include those segments of the polypeptide comprising a portion of the extracellular domain of the receptor. An example of a soluble VESPR polypeptide includes amino acids 1-944 of SEQ ID NO:2. In addition, truncated soluble VESPR proteins comprising less that the entire extracellular domain are included in the invention, *e.g.*, amino acids 35-944. Also encompassed within the present invention are the nucleic acid sequences encoding such useful VESPR polypeptides and polypeptide fragments. Particularly preferred nucleic acid sequences include the polynucleotide sequence of SEQ ID NO:1; and those segments of SEQ ID NO:1 that encode the soluble fragments of VESPR outlined above. The VESPR, its useful fragments, complements, variants, and combinations, such as fusion proteins as well as the nucleic acid sequences encoding these polypeptides are described in co-pending application SN 08/958,598 (specifically incorporated herein by reference, in its entirety). In embodiments of the present invention employing nucleic acid sequences, such as in "gene therapeutics," nucleic acid sequences encoding any of these semaphorin receptor polypeptides or their fragments can be used.

In a preferred embodiment, the present invention provides a pharmaceutical composition for the treatment of MDR phenotypic cells. This composition comprises an amount of a semaphorin or a semaphorin receptor polypeptide such that administration of the composition is effective to modulate the MDR phenotype of the target cells. Alternatively, in another aspect of the invention, the

composition further includes an amount of an expression vector including a nucleic acid sequence encoding a semaphorin, a semaphorin receptor, or a useful fragment of a semaphorin or semaphorin receptor, such that administration of the composition is effective to modulate the MDR phenotype of the target cell. This modulation may be to either promote or inhibit the development of multiple drug resistant cells.

An alternative embodiment of the present invention provides another pharmaceutical composition for the treatment of MDR phenotypic cells. In this aspect, the presently disclosed composition includes an amount of an agonist or antagonist for a semaphorin or a semaphorin receptor, such that administration of the composition is effective to promote or inhibit the development of MDR phenotype. Exemplary agonists or antagonists for semaphorins or semaphorin receptors include antibodies, such as, for example, either polyclonal or monoclonal antibodies, antigens and small molecules.

For example, a composition of the present invention can use a semaphorin antagonist, in the form of a soluble semaphorin receptor for example, to inhibit induction or activation of cellular efflux pumps. Use of such a composition allows one to decrease the ability of a cell to expel agents crossing the cell membrane, such as cytotoxic therapeutic agents. Alternatively or additionally, a composition of the present invention can include an antibody to a semaphorin receptor such as VESPR, which can function as either an antagonist or an agonist, or a small molecule agonist of a semaphorin receptor such as VESPR can be used.

In another embodiment, the present invention provides a pharmaceutical composition, for the treatment of cellular efflux-related disease states. In this aspect, the composition includes an amount of a semaphorin or semaphorin receptor such that administration of the composition is effective to modulate cellular efflux. Alternatively, in this aspect of the invention, the composition includes an amount of an expression vector including a nucleic acid sequence encoding a semaphorin, a semaphorin receptor, or encoding a useful fragment of a semaphorin or semaphorin receptor, such that administration of the composition is effective to modulate cellular efflux of the target cells. The active polypeptide or nucleic acid sequences of the composition used in this aspect of the invention may function to activate or up-regulate, or to inhibit or down-regulate, cellular efflux.

In an alternative embodiment, the present invention provides another composition for the treatment of cellular efflux-related disease states. In this embodiment, the disclosed composition includes an amount of an agonist or antagonist of a semaphorin or semaphorin receptor, such that administration of the composition is effective in activating or inhibiting cellular efflux in the target cell. Exemplary agonists or antagonists for semaphorins or semaphorin receptors include antibodies, such as, for example, either polyclonal or monoclonal antibodies; antigens and small molecules.

In another aspect, the present invention provides a method of modulating cellular efflux by administering to a cell an effective amount of a composition including a semaphorin or semaphorin receptor polypeptide such that cellular efflux is activated or inhibited. Alternatively, the present invention provides a method of modulating cellular efflux comprising administering to a cell, *via* an appropriate vector, an effective amount of a polynucleotide encoding a semaphorin, a semaphorin receptor, or a useful fragment of a semaphorin or semaphorin receptor, such that cellular efflux is activated or inhibited. Additionally, the presently disclosed methods of modulating cellular efflux, may comprise administering to a cell an effective amount of an agonist or antagonist of a semaphorin or semaphorin receptor such that cellular efflux is activated or inhibited. Exemplary useful agonists or antagonists include antibodies such as, for example, monoclonal or polyclonal antibodies, an antigen, or a small molecule. In a particularly preferred embodiment, the antibody used is an antibody to VESPR.

Pharmaceutical compositions and methods of the presently disclosed invention may be useful in the treatment of cellular efflux-related disease states such as multiple drug resistance; cancers, or other neoplastic diseases such as tumors, leukemia, lymphoma or other localized or metastatic conditions characterized by an abnormal proliferation of cells, generally due to cells continuing to replicate after the stimuli that initiated growth has ceased; cystic fibrosis arising from the treatment of a cell or group of cells with cytotoxic agents; auto-immune disorders; or acquired or genetically-based immunodeficiency disorders such as that resulting from the human immunodeficiency virus (HIV).

Formulation of any of the presently disclosed compositions for administration according to the disclosed methods can be done in any manner known to those of skill in the art. Such formulations will vary according to variables such as, for example, the needs of the formulator, the intended route of administration, the targeted disease or tissue, and the subject being treated. Specifically, unit doses may be formulated in multi-dose containers including additives such as a carrier, other excipients, and a preservative component.

The disclosed compositions may be formulated in a variety of concentrations in various vial sizes for various administration dosages. The presently disclosed compositions may also be in virtually any form including an aqueous solution, a suspension, a lyophilized form that may be reconstituted when appropriate, a gel, an aerosol, or any other form or state convenient for administration to treat the described disorders. The compositions as described herein may be formulated so that they are contained in a vial, bottle, tube, syringe, inhaler, transdermal patch, capsule or other container for single or multiple administrations.

In alternative embodiments, the presently disclosed compositions are formulated with or administered in conjunction with additional active agents such as chemotherapeutic agents, immune suppressants or radiation therapy. For example, agents that may be useful to co-formulate or

administer in conjunction with the disclosed compositions include virtually any chemotherapeutic or sensitizing agent such as cyclosporin, FK506, taxotere, doxorubicin, cis-platin, tamoxifen, i-phosphamide, or methotrexate, or variants of any of these compounds. Alternatively or additionally, the presently disclosed compositions may be further co-administered with an immune suppressant, such as a cytokine, IL-4, IL-12, , GM-CSF, G-CSF, M-CSF, α-interferon, β-interferon, or γ-interferon. The additional agents may be co-administered simultaneously or sequentially relative to the disclosed compositions and methods.

In another aspect, the present invention provides various assays and screening methods to identify substances that may be used to influence the MDR phenotype of a cell. For example, the present invention provides a method of detecting the ability of a test compound to affect the MDR phenotype of a cell, in which the following steps are used: (1) contacting a first cell with a test compound and a semaphorin or a semaphorin receptor, in the presence of a cytotoxic agent; (2) measuring the rate of death of the first cell; (3) observing the rate of death of a control cell in the absence of the test compound; and (4) comparing the rate of death of the first cell to the rate of death of the control cell. Upon comparison, a difference in the rate of cell death of the first cell relative to the control cell indicates that the test compound is an effector of MDR phenotype. In this manner, the effector can be identified as a substance that either promotes development of MDR phenotype or inhibits development of MDR phenotype. The affector can then be used therapeutically. Alternatively, the test compound may itself be a semaphorin or semaphorin receptor or fragment or antagonist or agonist thereof.

This method can be performed with a cytotoxic or sensitizing agent such as, for example, tamoxifen, cisplatin, doxorubicin, radiation, methotrexate, cyclosporin, taxotere, FK506, or i-phosphamide. Further, as with all compositions and methods of the present invention, the semaphorin or semaphorin receptor used in this method can be any known semaphorin or receptor polypeptide or useful fragment thereof, such as a fragment comprising the sema domain or the active domain of a semaphorin or semaphorin receptor. Additionally or alternatively, the presently disclosed method can be performed with any known semaphorin or semaphorin receptor, or fragment thereof being the test compound, or with an antibody to VESPR as the test compound.

In another aspect, the present invention provides a method of detecting the ability of a test compound to effect the MDR phenotype of a cell by modulating cellular efflux in the cell. Such a method would involve, for example, the following steps: (1) contacting a first cell with a test compound and a semaphorin or semaphorin receptor, in the presence of a dye; (2) measuring the net rate of influx of dye into the first cell; (3) observing the net rate of influx of dye into a control cell, in the absence of test compound comprising a semaphorin or semaphorin receptor, under otherwise identical conditions; and (4) comparing the net rate influx of dye into the first cell to the net rate of

influx of dye into the control cell. Upon comparison, a difference in the net rate of influx of dye into the first cell relative to the control cell indicates that the test compound is an effector of cellular efflux. In this manner, the effector can be identified as a substance that either promotes cellular efflux or inhibits cellular efflux and then can be used therapeutically. Alternatively, the test compound may itself be a semaphorin or semaphorin receptor or fragment or antagonist or agonist thereof.

Any dye may be used in the assays of the present invention. The dyes useful in such methods may be characterized by, for example, one or more of the following properties: lipophilic, cationic, fluorescent, and radioactive. Alternatively or additionally, the dye used in such methods can be a slow dye, a fast dye, acridine orange, BODIPY ceramide, SNARF-dextran, FITC-transferrin or BODIPY-transferrin.

As with all compositions and methods of the present invention, the semaphorin or semaphorin receptor used in this method can be any known semaphorin or semaphorin receptor polypeptide or useful fragment thereof, such as a fragment comprising the sema domain or the active domain of a semaphorin or semaphorin receptor. Additionally or alternatively, the presently disclosed method can be performed with any known semaphorin or semaphorin receptor, or fragment thereof being the test compound, or with an antibody to VESPR as the test compound.

In yet another aspect, the present invention provides pharmaceutical compositions and methods for the regulation of cellular-efflux, or MDR phenotype, by using the agent identified by the assays described herein. In this aspect of the invention, the modulating agent is effective to either inhibit or activate cellular efflux or development of drug resistance in a target cell.

IV. DETAILED DESCRIPTION OF THE INVENTION

Contrary to the results of Yamada *et al.*, who postulate that semaphorins function analogously to growth factors and may be involved in non-MDR drug resistance, the present invention teaches that semaphorins and semaphorin receptors can be used to influence the function of cellular efflux pumps in a variety of ways, including activation, inhibition, and promotion of stasis of the pumps and can be used to regulate MDR. The invention also teaches that, depending upon the specific semaphorin/receptor interaction, this influence can be inhibitory, and the capacity of a cell to eliminate cellular contents can be reduced, or the influence can be to promote cellular efflux and thereby facilitate expulsion of cellular contents. Accordingly, depending upon the effect, the disclosed semaphorin and semaphorin receptor compositions and methods are also useful: (1) to increase vulnerability or sensitivity of a cell to cytotoxic agents and thereby promote drug-induced cell death; (2) in identification or design of semaphorin or semaphorin receptor antagonists or agonists that might increase the sensitivity of a cell to a cytotoxic agent; (3) to promote cellular resistance to cytotoxic agents; or (4) in identification of semaphorin or semaphorin receptor agonists or antagonists that can be administered to cells to promote their resistance to various cytotoxic substances.

A. SEMAPHORIN AND SEMAPHORIN RECEPTOR POLYPEPTIDES

The terms "semaphorin" and "semaphorin polypeptide" are used interchangeably in the present invention. Semaphorins include proteins of the Semaphorin family and are either secreted or membrane-bound. Semaphorins have a well-conserved extracellular semaphorin (sema) domain. Generally, the sema domain is approximately 500 residues, but viral semaphorins themselves are only approximately 440 to 441 amino acids in length. It has been hypothesized that a 70 amino acid region with the sema domain is the active domain for semaphorin influence on certain cellular activities. *See* Koppel, *et al.* (1997). However it is not clear that this same region is the active site for all semaphorin activity. Accordingly, the present invention specifically contemplates the use of full-length semaphorin polypeptides, variants of these, and useful fragments of semaphorin polypeptides. Specific semaphorins and semaphorin fragments that are useful according to the present invention include, for example, the following semaphorins: AHV Sema; A39R; Sema I, including G-sema I and D-sema-I; Sema II; Sema III; Sema IV; DC Sema; CD100; Z SMF-7; Sema A; Sema B; Sema C; Sema D; Sema E; Sema H; Sema L; Sema W and Sema Y. Additionally, useful fragments of any semaphorin, such as the sema domain or the active domain may also be used according to the present invention. For additional semaphorins that can be used in the presently disclosed compositions and methods, *see* Bamberg, *et.al.* Cell, 97:551 and United States Patent No. 5,935,865 to Goodman *et al.* Nucleic acid sequences encoding the semaphorins or semaphorin fragments of the present invention, are also specifically contemplated to be useful in the disclosed compositions and methods.

"Semaphorin receptors" or "semaphorin receptor polypeptides" of the present invention are members of the Plexin family of semaphorin receptors. Plexins are membrane-bound polypeptides. Plexins contain a "sema" domain that is related to the sema domain of semaphorins themselves, part of which constitutes a series of two or three cystein repeat sequences in the extracellular domain of plexins. Plexins are distinct from semaphorins, however, in a variety of respects. For example, in their intracellular domain, plexins are strongly homologous throughout the family of plexins, and contain well-conserved amino acid motifs that are not found in semaphorins.

Semaphorin receptors of the present invention are those plexin polypeptide sequences that can interact with a semaphorin or a semaphorin fragment, to influence cellular efflux or development of MDR phenotype in a cell Exemplary semaphorin receptor polypeptides include full-length plexin receptor polypeptides as well as homologues or fragments, such as the soluble extra cellular domain or the sema domain of such plexin receptor polypeptides. Preferred semaphorin receptor polypeptides include the Viral Encoded Semaphorin Receptor (VESPR) or fragments thereof. As used herein, the term VESPR or VESPR polypeptide refers to any polypeptide functioning as a receptor for viral semaphorins, for human homologues to viral semaphorins, or for human semaphorins.

Additionally, useful soluble forms of the VESPR polypeptide include those segments of the polypeptide comprising a portion of the extracellular domain of the receptor. An example of a soluble VESPR polypeptide includes amino acids 1-944 of SEQ ID NO:2. In addition, truncated soluble VESPR proteins comprising less that the entire extracellular domain are included in the invention, *e.g.*, amino acids 35-944. Also encompassed within the present invention are the nucleic acid sequences encoding such useful VESPR polypeptides and polypeptide fragments. An exemplary Plexin receptor is the Viral Encoded Semaphorin Protein Receptor "VESPR," (described in copending patent application serial number 08/958,598). Specifically the amino acid sequence of SEQ ID NO:2 is useful as a semaphorin receptor polypeptide in the presently disclosed compositions and methods, as are the homologues and variants of polypeptides of SEQ ID NO:2. Nucleic acid sequences encoding the semaphorin receptors or receptor fragments are also within the scope of the presently disclosed compositions and methods. Particularly preferred nucleic acid sequences include the polynucleotide sequence of SEQ ID NO:1; and those segments of SEQ ID NO:1 that encode the soluble fragments of VESPR outlined above.

The semaphorin or semaphorin receptor polypeptides of the invention may be membrane bound or they may be secreted and thus soluble. Soluble polypeptides are capable of being secreted from the cells in which they are expressed. In general, soluble polypeptides may be identified (and distinguished from non-soluble membrane-bound counterparts) by separating intact cells which express the desired polypeptide from the culture medium, *e.g.*, by centrifugation, and assaying the medium (supernatant) for the presence of the desired polypeptide. The presence of polypeptide in the medium indicates that the polypeptide was secreted from the cells and thus is a soluble form of the protein.

In one embodiment, the soluble polypeptides and fragments thereof comprise all or part of the extracellular domain, but lack the transmembrane region that would cause retention of the polypeptide on a cell membrane. A soluble polypeptide may include the cytoplasmic domain, or a portion thereof, as long as the polypeptide is secreted from the cell in which it is produced.

In general, the use of soluble forms is advantageous for certain applications. Purification of the polypeptides from recombinant host cells is facilitated, since the soluble polypeptides are secreted from the cells. Further, soluble polypeptides are generally more suitable for intravenous administration.

The invention also provides polypeptides and fragments of the extracellular domain that retain a desired biological activity. Particular embodiments are directed to polypeptide fragments that retain the ability to interact with the semaphorin receptor or ligand to influence cellular efflux or the MDR phenotype of a cell. Such a fragment may be a soluble polypeptide, as described above. In another embodiment, the polypeptides and fragments advantageously include regions that are conserved in the

semaphorin family, in the case of semaphorins; or regions that are conserved in the plexin family in the case of the semaphorin receptors; or include the sema domain of either.

Also provided herein are polypeptide fragments comprising at least 20, or at least 30, contiguous amino acids of the sequence of SEQ ID NO:2. Fragments derived from the cytoplasmic domain find use in studies of signal transduction, and in regulating cellular processes associated with transduction of biological signals. Polypeptide fragments also may be employed as immunogens, in generating antibodies.

Naturally occurring variants as well as derived variants of the polypeptides and fragments are provided herein. Variants may exhibit amino acid sequences that are at least 80% identical. Also contemplated are embodiments in which a polypeptide or fragment comprises an amino acid sequence that is at least 90% identical, at least 95% identical, at least 98% identical, at least 99% identical, or at least 99.9% identical to the preferred polypeptide or fragment thereof. Percent identity can be determined by visual inspection and mathematical calculation. Alternatively, the percent identity of two protein sequences can be determined by comparing sequence information using the GAP computer program, based on the algorithm of Needleman and Wunsch (J. Mol. Bio. 48:443, 1970) and available from the University of Wisconsin Genetics Computer Group (UWGCG). The preferred default parameters for the GAP program include: (1) a scoring matrix, blosum62, as described by Henikoff and Henikoff (Proc. Natl. Acad. Sci. USA 89:10915, 1992); (2) a gap weight of 12; (3) a gap length weight of 4; and (4) no penalty for end gaps. Other programs used by one skilled in the art of sequence comparison may also be used.

The variants of the invention include, for example, those that result from alternate mRNA splicing events or from proteolytic cleavage. Alternate splicing of mRNA may, for example, yield a truncated but biologically active protein, such as a naturally occurring soluble form of the protein. Variations attributable to proteolysis include, for example, differences in the N- or C-termini upon expression in different types of host cells, due to proteolytic removal of one or more terminal amino acids from the protein (generally from 1-5 terminal amino acids). Proteins in which differences in amino acid sequence are attributable to genetic polymorphism (allelic variation among individuals producing the protein) are also contemplated herein.

Additional variants within the scope of the invention include polypeptides that may be modified to create derivatives thereof by forming covalent or aggregative conjugates with other chemical moieties, such as glycosyl groups, lipids, phosphate, acetyl groups and the like. Covalent derivatives may be prepared by linking the chemical moieties to functional groups on amino acid side chains or at the N-terminus or C-terminus of a polypeptide. Conjugates comprising diagnostic (detectable) or therapeutic agents attached thereto are contemplated herein, as discussed in more detail below.

Other derivatives include covalent or aggregative conjugates of the polypeptides with other proteins or polypeptides, such as by synthesis in recombinant culture as N-terminal or C-terminal fusions. Examples of fusion proteins are discussed below in connection with oligomers. Further, fusion proteins can comprise peptides added to facilitate purification and identification. Such peptides include, for example, poly-His or the antigenic identification peptides described in U.S. Patent No. 5,011,912 and in Hopp et al., *Bio/Technology* 6:1204, 1988. One such peptide is the FLAG® peptide, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys, which is highly antigenic and provides an epitope reversibly bound by a specific monoclonal antibody, enabling rapid assay and facile purification of expressed recombinant protein. A murine hybridoma designated 4E11 produces a monoclonal antibody that binds the FLAG® peptide in the presence of certain divalent metal cations, as described in U.S. Patent 5,011,912, hereby incorporated by reference. The 4E11 hybridoma cell line has been deposited with the American Type Culture Collection under accession no. HB 9259. Monoclonal antibodies that bind the FLAG® peptide are available from Eastman Kodak Co., Scientific Imaging Systems Division, New Haven, Connecticut.

Among the variant polypeptides provided herein are variants of native polypeptides that retain the native biological activity or the substantial equivalent thereof. One example is a variant that binds with essentially the same binding affinity as does the native form. Binding affinity can be measured by conventional procedures, *e.g.*, as described in U.S. Patent No. 5,512,457 and as set forth below.

Variants include polypeptides that are substantially homologous to the native form, but which have an amino acid sequence different from that of the native form because of one or more deletions, insertions or substitutions. Particular embodiments include, but are not limited to, polypeptides that comprise from one to ten deletions, insertions or substitutions of amino acid residues, when compared to a native sequence.

A given amino acid may be replaced, for example, by a residue having similar physiochemical characteristics. Examples of such conservative substitutions include substitution of one aliphatic residue for another, such as Ile, Val, Leu, or Ala for one another; substitutions of one polar residue for another, such as between Lys and Arg, Glu and Asp, or Gln and Asn; or substitutions of one aromatic residue for another, such as Phe, Trp, or Tyr for one another. Other conservative substitutions, *e.g.*, involving substitutions of entire regions having similar hydrophobicity characteristics, are well known.

Similarly, the DNAs of the invention include variants that differ from a native DNA sequence because of one or more deletions, insertions or substitutions, but that encode a biologically active polypeptide.

The invention further includes polypeptides of the invention with or without associated native-pattern glycosylation. Polypeptides expressed in yeast or mammalian expression systems (*e.g.*, COS-1 or COS-7 cells) can be similar to or significantly different from a native polypeptide in molecular

weight and glycosylation pattern, depending upon the choice of expression system. Expression of polypeptides of the invention in bacterial expression systems, such as *E. coli*, provides non-glycosylated molecules. Further, a given preparation may include multiple differentially glycosylated species of the protein. Glycosyl groups can be removed through conventional methods, in particular those utilizing glycopeptidase. In general, glycosylated polypeptides of the invention can be incubated with a molar excess of glycopeptidase (Boehringer Mannheim).

Correspondingly, similar DNA constructs that encode various additions or substitutions of amino acid residues or sequences, or deletions of terminal or internal residues or sequences are encompassed by the invention. For example, N-glycosylation sites in the polypeptide extracellular domain can be modified to preclude glycosylation, allowing expression of a reduced carbohydrate analog in mammalian and yeast expression systems. N-glycosylation sites in eukaryotic polypeptides are characterized by an amino acid triplet Asn-X-Y, wherein X is any amino acid except Pro and Y is Ser or Thr. Appropriate substitutions, additions, or deletions to the nucleotide sequence encoding these triplets will result in prevention of attachment of carbohydrate residues at the Asn side chain. Alteration of a single nucleotide, chosen so that Asn is replaced by a different amino acid, for example, is sufficient to inactivate an N-glycosylation site. Alternatively, the Ser or Thr can by replaced with another amino acid, such as Ala. Known procedures for inactivating N-glycosylation sites in proteins include those described in U.S. Patent 5,071,972 and EP 276,846, hereby incorporated by reference.

In another example of variants, sequences encoding Cys residues that are not essential for biological activity can be altered to cause the Cys residues to be deleted or replaced with other amino acids, preventing formation of incorrect intramolecular disulfide bridges upon folding or renaturation.

Other variants are prepared by modification of adjacent dibasic amino acid residues, to enhance expression in yeast systems in which KEX2 protease activity is present. EP 212,914 discloses the use of site-specific mutagenesis to inactivate KEX2 protease processing sites in a protein. KEX2 protease processing sites are inactivated by deleting, adding or substituting residues to alter Arg-Arg, Arg-Lys, and Lys-Arg pairs to eliminate the occurrence of these adjacent basic residues. Lys-Lys pairings are considerably less susceptible to KEX2 cleavage, and conversion of Arg-Lys or Lys-Arg to Lys-Lys represents a conservative and preferred approach to inactivating KEX2 sites.

Encompassed by the invention are oligomers or fusion proteins that contain semaphorin or semaphorin receptor polypeptides. Such oligomers may be in the form of covalently-linked or non-covalently-linked multimers, including dimers, trimers, or higher oligomers. As noted above, preferred polypeptides are soluble and thus these oligomers may comprise soluble polypeptides. In one aspect of the invention, the oligomers maintain the binding ability of the polypeptide components and provide therefor, bivalent, trivalent, etc., binding sites.

One embodiment of the invention is directed to oligomers comprising multiple polypeptides joined *via* covalent or non-covalent interactions between peptide moieties fused to the polypeptides. Such peptides may be peptide linkers (spacers), or peptides that have the property of promoting oligomerization. Leucine zippers and certain polypeptides derived from antibodies are among the peptides that can promote oligomerization of the polypeptides attached thereto, as described in more detail below.

As one alternative, an oligomer is prepared using polypeptides derived from immunoglobulins. Preparation of fusion proteins comprising certain heterologous polypeptides fused to various portions of antibody-derived polypeptides (including the Fc domain) has been described, *e.g.*, by Ashkenazi et al. (*PNAS USA* 88:10535, 1991); Byrn et al. (*Nature* 344:677, 1990); and Hollenbaugh and Aruffo ("Construction of Immunoglobulin Fusion Proteins", in *Current Protocols in Immunology*, Suppl. 4, pages 10.19.1 - 10.19.11, 1992).

One embodiment of the present invention is directed to a dimer comprising two fusion proteins created by fusing a polypeptide of the invention to an Fc polypeptide derived from an antibody. A gene fusion encoding the polypeptide/Fc fusion protein is inserted into an appropriate expression vector. Polypeptide/Fc fusion proteins are expressed in host cells transformed with the recombinant expression vector, and allowed to assemble much like antibody molecules, whereupon interchain disulfide bonds form between the Fc moieties to yield divalent molecules.

The term "Fc polypeptide" as used herein includes native and mutein forms of polypeptides made up of the Fc region of an antibody comprising any or all of the CH domains of the Fc region. Truncated forms of such polypeptides containing the hinge region that promotes dimerization are also included. Preferred polypeptides comprise an Fc polypeptide derived from a human IgG1 antibody.

One suitable Fc polypeptide, described in PCT application WO 93/10151 (hereby incorporated by reference), is a single chain polypeptide extending from the N-terminal hinge region to the native C-terminus of the Fc region of a human IgG1 antibody. Another useful Fc polypeptide is the Fc mutein described in U.S. Patent 5,457,035 and in Baum et al., (*EMBO J.* 13:3992-4001, 1994) incorporated herein by reference. The amino acid sequence of this mutein is identical to that of the native Fc sequence presented in WO 93/10151, except that amino acid 19 has been changed from Leu to Ala, amino acid 20 has been changed from Leu to Glu, and amino acid 22 has been changed from Gly to Ala. The mutein exhibits reduced affinity for Fc receptors.

The above-described fusion proteins comprising Fc moieties (and oligomers formed therefrom) offer the advantage of facile purification by affinity chromatography over Protein A or Protein G columns.

In other embodiments, the polypeptides of the invention may be substituted for the variable portion of an antibody heavy or light chain. If fusion proteins are made with both heavy and light

chains of an antibody, it is possible to form an oligomer with as many as four semaphorin or semaphorin receptor extracellular regions.

Alternatively, the oligomer is a fusion protein comprising multiple polypeptides, with or without peptide linkers (spacer peptides). Among the suitable peptide linkers are those described in U.S. Patents 4,751,180 and 4,935,233, which are hereby incorporated by reference. A DNA sequence encoding a desired peptide linker may be inserted between, and in the same reading frame as, the DNA sequences of the invention, using any suitable conventional technique. For example, a chemically synthesized oligonucleotide encoding the linker may be ligated between the sequences. In particular embodiments, a fusion protein comprises from two to four soluble semaphorin or semaphorin receptor polypeptides, separated by peptide linkers.

Another method for preparing the oligomers of the invention involves use of a leucine zipper. Leucine zipper domains are peptides that promote oligomerization of the proteins in which they are found. Leucine zippers were originally identified in several DNA-binding proteins (Landschulz et al., *Science* 240:1759, 1988), and have since been found in a variety of different proteins. Among the known leucine zippers are naturally occurring peptides and derivatives thereof that dimerize or trimerize. Zipper domains (also referred to herein as an oligomerizing, or oligomer-forming, domain) and their use are well-known in the art.

B. ASSAYS

The influence of semaphorins on cellular efflux may be used to control the development of MDR phenotypes of a cell or group of cells. For example, semaphorin polypeptides, or polynucleotides encoding semaphorin polypeptides may be administered to a cell or group of cells to stimulate or inhibit cellular efflux, to either induce, enhance, suppress or arrest the development of multiple drug resistance in the target cells. Identification of semaphorin-containing compositions that may be used in this manner may be carried out *via* a variety of assays known to those skilled in the art. Included in such assays are those that evaluate the ability of a semaphorin composition to influence cell survival rates in the presence of cytotoxic agents. Such an assay would involve, for example, the determination of sensitivities of tumor cells or cell lines to anticancer drugs in the presence and absence of a semaphorin. In these assays, one would determine a rate of cell death in the presence of the cytotoxic agent (such as doxorubicin, *etc.*) and then determine if the rate of cell death resulting from that agent is altered in the presence of a semaphorin.

Alternatively, one might monitor MDR protein-like activity in a cell by examining the ability of primary cells, or cells overexpressing MDR proteins, to efflux dyes in the presence and absence of a semaphorin. These types of assays are routine, and employ what are referred to as either "slow" or "fast" cellular dyes, that is, dyes that are typically lipophilic, or cationic. (*see, e.g.*, Lelong, *et al.*, 1991). One example of use of these dyes involves loading the dye into a cell at low temperatures, such

as 4 degrees (or on ice), and then examining the stained cells by flow cytometry. The cells will fluoresce depending on how much dye they take up; and, if loaded in the presence of an MDR efflux pump inhibitor such as verapamil or cyclosporin, or a semaphorin of the present invention, they may fluoresce more brightly than cells loaded in the absence of an MDR inhibitor. In this manner inhibitors of cellular efflux pumps can be identified. This assay may then also be taken a step further by transferring the cells loaded with dye to elevated temperature conditions, such as 37 degrees Centigrade, for a period of time, such as approximately three hours, at which time the cells are again examined on the flow cytometer, and compared to cells that were loaded with dye and held at cooler temperatures, such as the previously noted 4 degrees. At higher temperatures, cellular efflux pumps, including the MDR proteins, are quite active and can extrude the dye from the cell at a rapid rate. The ability of a semaphorin to influence this efflux can be measured by including the semaphorin in the assay during the efflux phase.

Yet another assay that may be used in the present invention involves examining intracellular pH, and the pH of intracellular compartments, in response to semaphorins. These types of assays again use fluorescent probes that target to the cytoplasm or to specific organelles, and exhibit fluorescence pattern changes as the pH changes. See, e.g. Altan, N *et al. J. Exp. Med.* 187:1583, Altan, N *et al. PNAS* 96:4432, Chen, Y *et al. JBC* 274:18364, Schindler, M. *et al. Biochemistry* 35:2811.. Dyes that are useful in such assays include dyes such as acridine orange (which targets acidic compartments and whose fluorescent wavelength and intensity depends on the pH of that organelle), BODIPY-ceramide (which targets the trans-golgi network), SNARF-dextrans of varying molecular weights (allowing one to target cytosol or nucleus), and FITC-transferrin or BODIPY-transferrin (which targets endocytic vesicles). These dyes are used to stain cells and then their fluorescence intensity and/or pattern is measured on a confocal microscope.

Another embodiment of the present invention provides a method of detecting the ability of the test compound to influence the MDR phenotype of a cell. In this aspect of the invention, the method includes contacting a first cell with a test compound including a semaphorin or semaphorin receptor in the presence of a cytotoxic agent. The method then involves measuring the rate of death of that first cell. Then the rate of death of a controlled cell is observed, with the control cell under similar conditions but in the absence of a test compound comprising a semaphorin or semaphorin receptor, and in the presence of a cytotoxic agent, which is the same agent administered to the first cell. The death rate of the first cell is then compared to the death rate of the control cell and the difference in the rate of cell death between the first cell and the control cell is indicative of an agent that influences development of multiple drug resistance phenotype. This agent may be one that functions to increase multiple drug resistance in the cell or to decrease multiple drug resistance in the cell.

In addition to those semaphorins listed in Section III above, specific semaphorins that may be tested according to this embodiment of the invention include A39R, DCSema, CD100, Sema III, Sema E, or active fragments of these semaphorins. Specific exemplary useful semaphorin receptors that may be used in these assays include VESPR. Alternatively, an agonist or antagonist to a semaphorin or semaphorin receptor may used according to this aspect of the invention. In a particularly preferred embodiment, an antibody to VESPR is used. Examples of cytotoxic agents that may be used in this method of detection include doxorubicin, radiation, tamoxifen, or any other compound known to have a cytotoxic effect on a cell.

In another aspect, the present invention provides a method of detecting the ability of a test compound to influence the MDR phenotype of a cell by modulating the cellular efflux of that cell. In this aspect, one example of such a method includes: (1) contacting a first cell with a test compound including a semaphorin or semaphorin receptor in the presence of a dye; (2) measuring the net rate of influx of dye into this first cell; and (3) observing the net rate of influx of dye into a control cell under similar conditions, but in the absence of a test compound comprising a semaphorin or semaphorin receptor. In this embodiment, the net rate of influx of dye is the rate of influx of dye relative to the rate of efflux, as measured by the amount of dye detected in the cell. The comparison will provide a difference in the net rate of influx of the dye such that influx of the dye into the first cell relative to the control cell is indicative of an agent that can influence cellular efflux. The test compound may function to either activate or up-regulate, or inhibit or down-regulate cellular efflux, either of which function may be detected through this method.

In addition to the semaphorins listed in Section III above, specific semaphorins that may be tested according to this embodiment of the invention include A39R, DCSema, CD100, Sema III, Sema E, or active fragments of these semaphorins. Specific exemplary useful semaphorin receptors that may be used in these assays include VESPR. Alternatively, an agonist or antagonist to a semaphorin or semaphorin receptor may used according to this aspect of the invention. In a particularly preferred embodiment, an antibody to VESPR is used.

Virtually any dye may be used in this method. Exemplary dyes include those which are characterized by one or more of the following properties: lipophilic, cationic, fluorescent, and radioactive. Specific exemplary dyes include a slow dye; a fast dye; acridine orange; various BODIPY dyes including specific ones such as 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene–3,5-dipropionic acid, BODIPY ceramide, and BODIPY-transferrin; seminaphthorhodafluors ("SNARF") –dextran; and Fluorescien isothiocyanate ("FITC")-transferrin.

C. Compounds and Methods for the Modulation of Cellular Efflux

Described below are methods and compositions employing semaphorins, semaphorin receptors, fragments of these, or the genes encoding them, for use in the promotion or suppression of

cellular efflux or for controlling development of MDR in a target cell or group of cells. It is specifically contemplated that such compositions and methods can be used to treat a cell or group of cells both *in vivo* and *in vitro*.

For example, such methods can comprise administering compounds which modulate cellular efflux, and thereby influence development of MDR phenotype or cellular efflux-related disease states. Administration of such compounds can be used to inhibit drug resistance thereby sensitizing cells to cytotoxic substances; to promote resistance to cytotoxic substances and protect against cytotoxic substances; or to the dysregulation of cellular efflux in cells that are unable to otherwise regulate themselves, such as those cells associated with diseases such as cystic fibrosis.

In addition to methods utilizing semaphorin or semaphorin receptor-encoding nucleic acid sequences, it is also useful to modulate cellular efflux by using the semaphorin or semaphorin receptor polypeptide, or polypeptide fragments. Another means of modulating cellular efflux or MDR phenotypes according to the present invention involves the use of any of the compounds identified through the assays set forth in Section B above.

When the actual nucleic acid sequences encoding the semaphorins; semaphorin receptors; or fragments of either that are disclosed in the present invention are delivered according to the methods described herein, it is advantageous to use a delivery mechanism so that the sequences will be incorporated into a cell for expression. Delivery systems that may advantageously be employed in the contemplated methods include the use of, for example, viral delivery systems such as retroviral and adenoviral vectors, as well as non-viral delivery systems. Such delivery systems are well known by those skilled in the art.

In one aspect of the invention, a retroviral delivery system may be employed. The retroviruses are a group of single-stranded RNA viruses characterized by an ability to convert their RNA to double-stranded DNA in infected cells by a process of reverse-transcription (Coffin, 1990). The resulting DNA then stably integrates into cellular chromosomes as a provirus and directs synthesis of viral proteins. The integration results in the retention of the viral gene sequences in the recipient cell and its descendants. The retroviral genome contains three genes - *gag*, *pol*, and *env* - that code for capsid proteins, polymerase enzyme, and envelope components, respectively. A sequence found upstream from the *gag* gene, termed ψ, functions as a signal for packaging of the genome into virions. Two long terminal repeat (LTR) sequences are present at the 5' and 3' ends of the viral genome. These contain strong promoter and enhancer sequences and are also required for integration in the host cell genome (Coffin, 1990).

In order to construct a retroviral vector, a nucleic acid encoding a Grb2 or Crkl antisense construct is inserted into the viral genome in the place of certain viral sequences to produce a virus that is replication-defective. In order to produce virions, a packaging cell line containing the *gag*, pol and *env* genes but without the LTR and ψ components is constructed (Mann *et al.*, 1983). When a recombinant

plasmid containing an inserted DNA, together with the retroviral LTR and ψ sequences, is introduced into this cell line (by calcium phosphate precipitation for example), the ψ sequence allows the RNA transcript of the recombinant plasmid to be packaged into viral particles, which are then secreted into the culture media (Nicolas and Rubenstein, 1988; Temin, 1986; Mann *et al.*, 1983). The media containing the recombinant retroviruses is then collected, optionally concentrated, and used for gene transfer. Retroviral vectors are able to infect a broad variety of cell types. However, integration and stable expression require the division of host cells (Paskind *et al.*, 1975).

Alternatively, an adenoviral delivery system may be employed. Human adenoviruses are double-stranded DNA tumor viruses with genome sizes of approximate 36 kB (Tooze, 1981). As a model system for eukaryotic gene expression, adenoviruses have been widely studied and well characterized, which makes them an attractive system for development of adenovirus as a gene transfer system. This group of viruses is easy to grow and manipulate, and they exhibit a broad host range *in vitro* and *in vivo*. In lytically infected cells, adenoviruses are capable of shutting off host protein synthesis, directing cellular machineries to synthesize large quantities of viral proteins, and producing copious amounts of virus.

The E1 region of the genome includes E1A and E1B which encode proteins responsible for transcription regulation of the viral genome, as well as a few cellular genes. E2 expression, including E2A and E2B, allows synthesis of viral replicative functions, *e.g.* DNA-binding protein, DNA polymerase, and a terminal protein that primes replication. E3 gene products prevent cytolysis by cytotoxic T cells and tumor necrosis factor and appear to be important for viral propagation. Functions associated with the E4 proteins include DNA replication, late gene expression, and host cell shutoff. The late gene products include most of the virion capsid proteins, and these are expressed only after most of the processing of a single primary transcript from the major late promoter has occurred. The major late promoter (MLP) exhibits high efficiency during the late phase of the infection (Stratford-Perricaudet and Perricaudet, 1991).

As only a small portion of the viral genome appears to be required *in cis* (Tooze, 1981), adenovirus-derived vectors offer excellent potential for the substitution of large DNA fragments when used in connection with cell lines such as 293 cells. Ad5-transformed human embryonic kidney cell lines (Graham, *et al.*, 1977) have been developed to provide the essential viral proteins *in trans*.

Particular advantages of an adenovirus system for delivering foreign proteins to a cell include (i) the ability to substitute relatively large pieces of viral DNA by foreign DNA; (ii) the structural stability of recombinant adenoviruses; (iii) the safety of adenoviral administration to humans; and (iv) lack of any known association of adenoviral infection with cancer or malignancies; (v) the ability to obtain high titers of the recombinant virus; and (vi) the high infectivity of adenovirus.

Further advantages of adenovirus vectors over retroviruses include the higher levels of gene expression. Additionally, adenovirus replication is independent of host gene replication, unlike retroviral

sequences. Because adenovirus transforming genes in the E1 region can be readily deleted and still provide efficient expression vectors, oncogenic risk from adenovirus vectors is thought to be negligible (Grunhaus & Horwitz, 1992).

In general, adenovirus gene transfer systems are based upon recombinant, engineered adenovirus which is rendered replication-incompetent by deletion of a portion of its genome, such as E1, and yet still retains its competency for infection. Sequences encoding relatively large foreign proteins can be expressed when additional deletions are made in the adenovirus genome. For example, adenoviruses deleted in both E1 and E3 regions are capable of carrying up to 10 kB of foreign DNA and can be grown to high titers in 293 cells (Stratford-Perricaudet and Perricaudet, 1991). Surprisingly persistent expression of transgenes following adenoviral infection has also been reported.

Other viral vectors may be employed as expression constructs in the present invention. Vectors derived from viruses such as vaccinia virus (Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988) adeno-associated virus (AAV) (Ridgeway, 1988; Baichwal and Sugden, 1986; Hermonat and Muzycska, 1984) and herpesviruses may be employed. They offer several attractive features for various mammalian cells (Friedmann, 1989; Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988; Horwich *et al.*, 1990).

With the recent recognition of defective hepatitis B viruses, new insight was gained into the structure-function relationship of different viral sequences. *In vitro* studies showed that the virus could retain the ability for helper-dependent packaging and reverse transcription despite the deletion of up to 80% of its genome (Horwich *et al.*, 1990). This suggested that large portions of the genome could be replaced with foreign genetic material. The hepatotropism and persistence (integration) were particularly attractive properties for liver-directed gene transfer. Chang *et al.* recently introduced the chloramphenicol acetyltransferase (CAT) gene into duck hepatitis B virus genome in the place of the polymerase, surface, and pre-surface coding sequences. It was cotransfected with wild-type virus into an avian hepatoma cell line. Culture media containing high titers of the recombinant virus were used to infect primary duckling hepatocytes. Stable CAT gene expression was detected for at least 24 days after transfection (Chang *et al.*, 1991).

In yet another aspect, non-viral vectors may be used according to the presently disclosed methods. Several non-viral methods for the transfer of expression vectors into cultured mammalian cells also are contemplated by the present invention. These include calcium phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe *et al.*, 1990) DEAE-dextran (Gopal, 1985), electroporation (Tur-Kaspa *et al.*, 1986; Potter *et al.*, 1984), direct microinjection (Harland and Weintraub, 1985), DNA-loaded liposomes (Nicolau and Sene, 1982; Fraley *et al.*, 1979) and lipofectamine-DNA complexes, cell sonication (Fechheimer *et al.*, 1987), gene bombardment using high velocity microprojectiles (Yang *et al.*, 1990), polycations (Boussif *et al.*, 1995) and receptor-mediated transfection

(Wu and Wu, 1987; Wu and Wu, 1988). Some of these techniques may be successfully adapted for *in vivo* or *ex vivo* use.

In one embodiment of the invention, the expression construct may simply consist of naked recombinant vector. Transfer of the construct may be performed by any of the methods mentioned above which physically or chemically permeabilize the cell membrane. For example, Dubensky *et al.* (1984) successfully injected polyomavirus DNA in the form of $CaPO_4$ precipitates into liver and spleen of adult and newborn mice demonstrating active viral replication and acute infection. Benvenisty and Neshif (1986) also demonstrated that direct intraperitoneal injection of $CaPO_4$ precipitated plasmids results in expression of the transfected genes. It is envisioned that DNA encoding an Grb2 or Crkl construct may also be transferred in a similar manner *in vivo*.

Another embodiment of the invention for transferring a naked DNA expression vector into cells may involve particle bombardment. This method depends on the ability to accelerate DNA coated microprojectiles to a high velocity allowing them to pierce cell membranes and enter cells without killing them (Klein *et al.*, 1987). Several devices for accelerating small particles have been developed. One such device relies on a high voltage discharge to generate an electrical current, which in turn provides the motive force (Yang *et al.*, 1990). The microprojectiles used have consisted of biologically inert substances such as tungsten or gold beads.

Selected organs including the liver, skin, and muscle tissue of rats and mice have been bombarded *in vivo* (Yang *et al.*, 1990; Zelenin *et al.*, 1991). This may require surgical exposure of the tissue or cells, to eliminate any intervening tissue between the gun and the target organ. DNA encoding a Grb2 or Crkl construct may be delivered via this method.

Alternatively, the degree of cellular efflux in a cell may be influenced by administering a compound identified *via* one of the assays described above, that increases or decreases the rate of cellular efflux or development of MDR phenotype.

D. FORMULATION AND ADMINISTRATION OF THE DISCLOSED COMPOSITIONS

The formulations described herein may be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose or polyoxyethylenesorbitans. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride as described above. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate or gelatin. Other agents that may be employed include, but are not limited to lecithin, urea, ethylene oxide, propylene oxide, hydroxypropylcellulose, methylcellulose, or polyethylene glycol.

Aqueous compositions (inocula) as described herein may include an effective amount of a desired pharmacologically active agent dissolved or dispersed in a pharmaceutically acceptable aqueous medium. Such compositions are also referred to as inocula. The use of pharmaceutically

acceptable carrier media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions as described above.

A semaphorin used in the present invention may be formulated into a composition in a neutral or salt form. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and those that are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

Such compositions of the present invention can be, alternatively, complexed with polyethylene glycol (PEG), metal ions, or incorporated into polymeric compounds such as polyacetic acid, polyglycolic acid, hydrogels, dextran, *etc.* or incorporated into liposomes, microemulsions, micelles, unilamellar or multilamellar vesicles, erythrocyte ghosts or sphereoblasts. Such compositions will influence the physical state, solubility, stability, rate of *in vivo* release, and rate of *in vivo* clearance, and are thus chosen according to the intended application.

The therapeutic compositions of the present invention are advantageously administered in the form of injectable compositions either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. Alternatively, the compositions of the present invention may be administered as inhalants in an aerosolized form. Depending upon the needs of the formulator, administrator, or the subject of the treatment, the presently disclosed compositions may take virtually any form including liquid, suspension, aerosol, emulsion, solution, oil, mixture, cream, ointment, gel, suppository, semi-solid, aerosol, powder, lyophilized form that may be reconstituted when appropriate, tablet, capsule or any other form or state convenient for administration to treat the described disorders. A typical composition comprises a pharmaceutically acceptable carrier.

The presently disclosed compositions and methods may utilize both oral and non-oral administration routes to influence the target cell or cells including, for example, by injection *via* the intradermal, subcutaneous, and intravenous routes; by transdermal delivery; by inhalation or buccal delivery, or by ingestion of tablets or capsules. For example, local or regional delivery of compounds to a cell or cells can be by injection into the tissue, injection into the vasculature or lymphatics to effect regional infusion, inhalation, or regional perfusion by use of an extracorporeal circuit. Administration in a targeted fashion is useful to, for example, more effectively eliminate neoplastic cells, while minimizing the adverse effects of chemotherapy on healthy cells. For example, an inhibitor of cellular efflux can be directly administered, according to the methods disclosed herein, to

neoplastic cells such as tumor cells, to prevent their development of MDR and thereby promote their susceptibility to chemotherapeutic or otherwise cytotoxic agents, while simultaneously administering to healthy cells a promoter of cellular-efflux to prevent their destruction by cytotoxic agents.

The optimal daily dose of semaphorin, semaphorin receptor such as VESPR or soluble VESPR, or of an agonist or antagonist of one of these, alone or in combination, useful for the purposes of the present invention is determined by methods known in the art. For example, dosages can be determined based on the severity of the disease or condition being treated, the condition of the subject to whom treatment is being given, the desired degree of therapeutic response, and any concomitant therapies being administered to the subject. Ordinarily, however, administration will be such that a serum level of between about 100ng/ml to about 100μg/ml of semaphorin, semaphorin receptor, or agonist or antagonist of either, is achieved. Preferred doses will achieve blood serum levels of between 500ng/ml and 1μg/ml. The dose can be administered in a single or multiple dosage regimen, or may be by a method that allows for a continuous release of relatively small amounts of the active ingredient from a single dosage unit, such as by a transdermal patch or ingested extended release capsule, over the course of one or more days.

To determine when inhibition or retardation of the various target diseases or conditions, or when amelioration, regression or destruction of the targeted diseases or conditions has been achieved, any of the following can be considered: improvement in patient condition or quality of life; increased longevity of life; decreased pain; decreased severity of symptoms of the targeted disease or condition; retardation of abnormal tissue growth or metastases such as in the case of suppression of development of MDR in cells being targeted for cancer chemotherapeutic disease; an increase in desired tissue growth or viability in the case or promotion of drug resistance in healthy tissue and cells; and the like. Any of these endpoints as well as others may be considered to determine the effectiveness of the therapy, and may be measured or determined by patient self-evaluation; objective screening; or by diagnostic testing such as by X-ray, CT or PET scanning or the like.

The compositions as described herein may be formulated so that they are contained in a vial, bottle, tube, syringe inhaler or other container for single or multiple administrations. Such containers may be made of glass or a polymer material such as polypropylene, polyethylene, or polyvinylchloride, for example. Preferred containers may include a seal, or other closure system, such as a rubber stopper that may be penetrated by a needle in order to withdraw a single dose and then re-seal upon removal of the needle. All such containers for injectable liquids, lyophilized formulations, reconstituted lyophilized formulations or reconstitutable powders for injection known in the art or for the administration of aerosolized compositions are contemplated for use in the presently disclosed compositions and methods.

In alternative embodiments, the presently disclosed compositions are administered in conjunction, either simultaneously or sequentially, with additional active agents such as an immunosuppressant, cell sensitizer, or other chemotherapeutic agent including a cancer chemotherapeutic agent. Exemplary agents to be used in combination with the presently disclosed compositions include cyclosporin, tamoxifen, FK506, taxotere, doxorubicin, cis-platin, I-phosphamide, or methotrexate.

REFERENCES

Altan, *et al.*, *J. Exp. Med.* 187:1583

Altan, *et al.*, *PNAS* 96:4432

Chen, *et al.*, *JBC* 274:18364

Kim *et al.*, Blood, 91:4106-4117 (1998)

Lelong, *et al.*, Molecular Pharmacology 40:490 (1991).

Schindler, *et al.*, *Biochemistry* 35:2811

Weisburg et al, J. Biol Chem, 274, 10877-88 (1999)

Yamada *et.al.*, PNAS 94:14713-14718 (1997)

Zaman, G.J.R. *et.al.*, PNAS USA 91:8822 (1994)

CLAIMS

What is claimed is:

1. Use of a semaphorin, a semaphorin receptor, or an agonist or antagonist of a semaphorin or semaphorin receptor, in the manufacture of a medicament for multiple drug resistance, cystic fibrosis or immunodeficiency disease.

INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4707 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..4707

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
ATG GAG GTC TCC CGG AGG AAG GCG CCG CCG CGC CCC CCG CGC CCC GCA      48
Met Glu Val Ser Arg Arg Lys Ala Pro Pro Arg Pro Pro Arg Pro Ala
 1               5                  10                  15

GCG CCA CTG CCC CTG CTC GCC TAT CTG CTG GCA CTG GCG GCT CCC GGC      96
Ala Pro Leu Pro Leu Leu Ala Tyr Leu Leu Ala Leu Ala Ala Pro Gly
            20                  25                  30

CGG GGC GCG GAC GAG CCC GTG TGG CGG TCG GAG CAA GCC ATC GGA GCC     144
Arg Gly Ala Asp Glu Pro Val Trp Arg Ser Glu Gln Ala Ile Gly Ala
        35                  40                  45

ATC GCG GCG AGC CAG GAG GAC GGC GTG TTT GTG GCG AGC GGC AGC TGC     192
Ile Ala Ala Ser Gln Glu Asp Gly Val Phe Val Ala Ser Gly Ser Cys
    50                  55                  60

CTG GAC CAG CTG GAC TAC AGC CTG GAG CAC AGC CTC TCG CGC CTG TAC     240
Leu Asp Gln Leu Asp Tyr Ser Leu Glu His Ser Leu Ser Arg Leu Tyr
65                  70                  75                  80

CGG GAC CAA GCG GGC AAC TGC ACA GAG CCG GTC TCG CTG GCG CCC CCC     288
Arg Asp Gln Ala Gly Asn Cys Thr Glu Pro Val Ser Leu Ala Pro Pro
                85                  90                  95

GCG CGG CCC CGG CCC GGG AGC AGC TTC AGC AAG CTG CTG CTG CCC TAC     336
Ala Arg Pro Arg Pro Gly Ser Ser Phe Ser Lys Leu Leu Leu Pro Tyr
            100                 105                 110

CGC GAG GGG GCG GCC GGC CTC GGG GGG CTG CTG CTC ACC GGC TGG ACC     384
Arg Glu Gly Ala Ala Gly Leu Gly Gly Leu Leu Leu Thr Gly Trp Thr
        115                 120                 125

TTC GAC CGG GGC GCC TGC GAG GTG CGG CCC CTG GGC AAC CTG AGC CGC     432
```

```
Phe Asp Arg Gly Ala Cys Glu Val Arg Pro Leu Gly Asn Leu Ser Arg
    130                 135                 140

AAC TCC CTG CGC AAC GGC ACC GAG GTG GTG TCG TGC CAC CCG CAG GGC      480
Asn Ser Leu Arg Asn Gly Thr Glu Val Val Ser Cys His Pro Gln Gly
145                 150                 155                 160

TCG ACG GCC GGC GTG GTG TAC CGC GCG GGC CGG AAC AAC CGC TGG TAC      528
Ser Thr Ala Gly Val Val Tyr Arg Ala Gly Arg Asn Asn Arg Trp Tyr
                165                 170                 175

CTG GCG GTG GCC GCC ACC TAC GTG CTG CCT GAG CCG GAG ACG GCG AGC      576
Leu Ala Val Ala Ala Thr Tyr Val Leu Pro Glu Pro Glu Thr Ala Ser
            180                 185                 190

CGC TGC AAC CCC GCG GCA TCC GAC CAC GAC ACG GCC ATC GCG CTC AAG      624
Arg Cys Asn Pro Ala Ala Ser Asp His Asp Thr Ala Ile Ala Leu Lys
        195                 200                 205

GAC ACG GAG GGG CGC AGC CTG GCC ACG CAG GAG CTG GGG CGC CTC AAG      672
Asp Thr Glu Gly Arg Ser Leu Ala Thr Gln Glu Leu Gly Arg Leu Lys
    210                 215                 220

CTG TGC GAG GGC GCG GGC AGC CTG CAC TTC GTG GAC GCC TTT CTC TGG      720
Leu Cys Glu Gly Ala Gly Ser Leu His Phe Val Asp Ala Phe Leu Trp
225                 230                 235                 240

AAC GGC AGC ATC TAC TTC CCC TAC TAC CCC TAC AAC TAT ACG AGC GGC      768
Asn Gly Ser Ile Tyr Phe Pro Tyr Tyr Pro Tyr Asn Tyr Thr Ser Gly
                245                 250                 255

GCT GCC ACC GGC TGG CCC AGC ATG GCG CGC ATC GCG CAG AGC ACC GAG      816
Ala Ala Thr Gly Trp Pro Ser Met Ala Arg Ile Ala Gln Ser Thr Glu
            260                 265                 270

GTG CTG TTC CAG GGC CAG GCA TCC CTC GAC TGC GGC CAC GGC CAC CCC      864
Val Leu Phe Gln Gly Gln Ala Ser Leu Asp Cys Gly His Gly His Pro
        275                 280                 285

GAC GGC CGC CGC CTG CTC CTC TCC TCC AGC CTA GTG GAG GCC CTG GAC      912
Asp Gly Arg Arg Leu Leu Leu Ser Ser Ser Leu Val Glu Ala Leu Asp
    290                 295                 300

GTC TGG GCG GGA GTG TTC AGC GCG GCC GCT GGA GAG GGC CAG GAG CGG      960
Val Trp Ala Gly Val Phe Ser Ala Ala Ala Gly Glu Gly Gln Glu Arg
305                 310                 315                 320

CGC TCC CCC ACC ACC ACG GCG CTC TGC CTC TTC AGA ATG AGT GAG ATC     1008
Arg Ser Pro Thr Thr Thr Ala Leu Cys Leu Phe Arg Met Ser Glu Ile
                325                 330                 335

CAG GCG CGC GCC AAG AGG GTC AGC TGG GAC TTC AAG ACG GCC GAG AGC     1056
Gln Ala Arg Ala Lys Arg Val Ser Trp Asp Phe Lys Thr Ala Glu Ser
            340                 345                 350
```

```
CAC TGC AAA GAA GGG GAT CAA CCT GAA AGA GTC CAA CCA ATC GCA TCA      1104
His Cys Lys Glu Gly Asp Gln Pro Glu Arg Val Gln Pro Ile Ala Ser
        355                 360                 365

TCT ACC TTG ATC CAT TCC GAC CTG ACA TCC GTT TAT GGC ACC GTG GTA      1152
Ser Thr Leu Ile His Ser Asp Leu Thr Ser Val Tyr Gly Thr Val Val
    370                 375                 380

ATG AAC AGG ACT GTT TTA TTC TTG GGG ACT GGA GAT GGC CAG TTA CTT      1200
Met Asn Arg Thr Val Leu Phe Leu Gly Thr Gly Asp Gly Gln Leu Leu
385                 390                 395                 400

AAG GTT ATT CTT GGT GAG AAT TTG ACT TCA AAT TGT CCA GAG GTT ATC      1248
Lys Val Ile Leu Gly Glu Asn Leu Thr Ser Asn Cys Pro Glu Val Ile
                405                 410                 415

TAT GAA ATT AAA GAA GAG ACA CCT GTT TTC TAC AAA CTC GTT CCT GAT      1296
Tyr Glu Ile Lys Glu Glu Thr Pro Val Phe Tyr Lys Leu Val Pro Asp
            420                 425                 430

CCT GTG AAG AAT ATC TAC ATT TAT CTA ACA GCT GGG AAA GAG GTG AGG      1344
Pro Val Lys Asn Ile Tyr Ile Tyr Leu Thr Ala Gly Lys Glu Val Arg
        435                 440                 445

AGA ATT CGT GTT GCA AAC TGC AAT AAA CAT AAA TCC TGT TCG GAG TGT      1392
Arg Ile Arg Val Ala Asn Cys Asn Lys His Lys Ser Cys Ser Glu Cys
    450                 455                 460

TTA ACA GCC ACA GAC CCT CAC TGC GGT TGG TGC CAT TCG CTA CAA AGG      1440
Leu Thr Ala Thr Asp Pro His Cys Gly Trp Cys His Ser Leu Gln Arg
465                 470                 475                 480

TGC ACT TTT CAA GGA GAT TGT GTA CAT TCA GAG AAC TTA GAA AAC TGG      1488
Cys Thr Phe Gln Gly Asp Cys Val His Ser Glu Asn Leu Glu Asn Trp
                485                 490                 495

CTG GAT ATT TCG TCT GGA GCA AAA AAG TGC CCT AAA ATT CAG ATA ATT      1536
Leu Asp Ile Ser Ser Gly Ala Lys Lys Cys Pro Lys Ile Gln Ile Ile
            500                 505                 510

CGA AGC AGT AAA GAA AAG ACT ACA GTG ACT ATG GTG GGA AGC TTC TCT      1584
Arg Ser Ser Lys Glu Lys Thr Thr Val Thr Met Val Gly Ser Phe Ser
        515                 520                 525

CCA AGA CAC TCA AAG TGC ATG GTG AAG AAT GTG GAC TCT AGC AGG GAG      1632
Pro Arg His Ser Lys Cys Met Val Lys Asn Val Asp Ser Ser Arg Glu
    530                 535                 540

CTC TGC CAG AAT AAA AGT CAG CCC AAC CGG ACC TGC ACC TGT AGC ATC      1680
Leu Cys Gln Asn Lys Ser Gln Pro Asn Arg Thr Cys Thr Cys Ser Ile
545                 550                 555                 560

CCA ACC AGA GCA ACC TAC AAA GAT GTT TCA GTT GTC AAC GTG ATG TTC      1728
Pro Thr Arg Ala Thr Tyr Lys Asp Val Ser Val Val Asn Val Met Phe
                565                 570                 575
```

```
TCC TTC GGT TCT TGG AAT TTA TCA GAC AGA TTC AAC TTT ACC AAC TGC      1776
Ser Phe Gly Ser Trp Asn Leu Ser Asp Arg Phe Asn Phe Thr Asn Cys
            580                 585                 590

TCA TCA TTA AAA GAA TGC CCA GCA TGC GTA GAA ACT GGC TGC GCG TGG      1824
Ser Ser Leu Lys Glu Cys Pro Ala Cys Val Glu Thr Gly Cys Ala Trp
        595                 600                 605

TGT AAA AGT GCA AGA AGG TGT ATC CAC CCC TTC ACA GCT TGC GAC CCT      1872
Cys Lys Ser Ala Arg Arg Cys Ile His Pro Phe Thr Ala Cys Asp Pro
    610                 615                 620

TCT GAT TAT GAG AGA AAC CAG GAA CAG TGT CCA GTG GCT GTC GAG AAG      1920
Ser Asp Tyr Glu Arg Asn Gln Glu Gln Cys Pro Val Ala Val Glu Lys
625                 630                 635                 640

ACA TCA GGA GGA GGA AGA CCC AAG GAG AAC AAG GGG AAC AGA ACC AAC      1968
Thr Ser Gly Gly Gly Arg Pro Lys Glu Asn Lys Gly Asn Arg Thr Asn
                645                 650                 655

CAG GCT TTA CAG GTC TTC TAC ATT AAG TCC ATT GAG CCA CAG AAA GTA      2016
Gln Ala Leu Gln Val Phe Tyr Ile Lys Ser Ile Glu Pro Gln Lys Val
            660                 665                 670

TCG ACA TTA GGG AAA AGC AAC GTG ATA GTA ACG GGA GCA AAC TTT ACC      2064
Ser Thr Leu Gly Lys Ser Asn Val Ile Val Thr Gly Ala Asn Phe Thr
        675                 680                 685

CGG GCA TCG AAC ATC ACA ATG ATC CTG AAA GGA ACC AGT ACC TGT GAT      2112
Arg Ala Ser Asn Ile Thr Met Ile Leu Lys Gly Thr Ser Thr Cys Asp
    690                 695                 700

AAG GAT GTG ATA CAG GTT AGC CAT GTG CTA AAT GAC ACC CAC ATG AAA      2160
Lys Asp Val Ile Gln Val Ser His Val Leu Asn Asp Thr His Met Lys
705                 710                 715                 720

TTC TCT CTT CCA TCA AGC CGG AAA GAA ATG AAG GAT GTG TGT ATC CAG      2208
Phe Ser Leu Pro Ser Ser Arg Lys Glu Met Lys Asp Val Cys Ile Gln
                725                 730                 735

TTT GAT GGT GGG AAC TGC TCT TCT GTG GGA TCC TTA TCC TAC ATT GCT      2256
Phe Asp Gly Gly Asn Cys Ser Ser Val Gly Ser Leu Ser Tyr Ile Ala
            740                 745                 750

CTG CCA CAT TGT TCC CTT ATA TTT CCT GCT ACC ACC TGG ATC AGT GGT      2304
Leu Pro His Cys Ser Leu Ile Phe Pro Ala Thr Thr Trp Ile Ser Gly
        755                 760                 765

GGT CAA AAT ATA ACC ATG ATG GGC AGA AAT TTT GAT GTA ATT GAC AAC      2352
Gly Gln Asn Ile Thr Met Met Gly Arg Asn Phe Asp Val Ile Asp Asn
    770                 775                 780

TTA ATC ATT TCA CAT GAA TTA AAA GGA AAC ATA AAT GTC TCT GAA TAT      2400
Leu Ile Ile Ser His Glu Leu Lys Gly Asn Ile Asn Val Ser Glu Tyr
```

```
785                 790                 795                 800

TGT GTG GCG ACT TAC TGC GGG TTT TTA GCC CCC AGT TTA AAG AGT TCA      2448
Cys Val Ala Thr Tyr Cys Gly Phe Leu Ala Pro Ser Leu Lys Ser Ser
                805                 810                 815

AAA GTG CGC ACG AAT GTC ACT GTG AAG CTG AGA GTA CAA GAC ACC TAC      2496
Lys Val Arg Thr Asn Val Thr Val Lys Leu Arg Val Gln Asp Thr Tyr
            820                 825                 830

TTG GAT TGT GGA ACC CTG CAG TAT CGG GAG GAC CCC AGA TTC ACG GGG      2544
Leu Asp Cys Gly Thr Leu Gln Tyr Arg Glu Asp Pro Arg Phe Thr Gly
        835                 840                 845

TAT CGG GTG GAA TCC GAG GTG GAC ACA GAA CTG GAA GTG AAA ATT CAA      2592
Tyr Arg Val Glu Ser Glu Val Asp Thr Glu Leu Glu Val Lys Ile Gln
    850                 855                 860

AAA GAA AAT GAC AAC TTC AAT ATT TCC AAA AAA GAC ATT GAA ATT ACT      2640
Lys Glu Asn Asp Asn Phe Asn Ile Ser Lys Lys Asp Ile Glu Ile Thr
865                 870                 875                 880

CTC TTC CAT GGG GAA AAT GGG CAA TTA AAT TGC AGT TTT GAA AAT ATT      2688
Leu Phe His Gly Glu Asn Gly Gln Leu Asn Cys Ser Phe Glu Asn Ile
                885                 890                 895

ACT AGA AAT CAA GAT CTT ACC ACC ATC CTT TGC AAA ATT AAA GGC ATC      2736
Thr Arg Asn Gln Asp Leu Thr Thr Ile Leu Cys Lys Ile Lys Gly Ile
            900                 905                 910

AAG ACT GCA AGC ACC ATT GCC AAC TCT TCT AAG AAA GTT CGG GTC AAG      2784
Lys Thr Ala Ser Thr Ile Ala Asn Ser Ser Lys Lys Val Arg Val Lys
        915                 920                 925

CTG GGA AAC CTG GAG CTC TAC GTC GAG CAG GAG TCA GTT CCT TCC ACA      2832
Leu Gly Asn Leu Glu Leu Tyr Val Glu Gln Glu Ser Val Pro Ser Thr
    930                 935                 940

TGG TAT TTT CTG ATT GTG CTC CCT GTC TTG CTA GTG ATT GTC ATT TTT      2880
Trp Tyr Phe Leu Ile Val Leu Pro Val Leu Leu Val Ile Val Ile Phe
945                 950                 955                 960

GCG GCC GTG GGG GTG ACC AGG CAC AAA TCG AAG GAG CTG AGT CGC AAA      2928
Ala Ala Val Gly Val Thr Arg His Lys Ser Lys Glu Leu Ser Arg Lys
                965                 970                 975

CAG AGT CAA CAA CTA GAA TTG CTG GAA AGC GAG CTC CGG AAA GAG ATA      2976
Gln Ser Gln Gln Leu Glu Leu Leu Glu Ser Glu Leu Arg Lys Glu Ile
            980                 985                 990

CGT GAC GGC TTT GCT GAG CTG CAG ATG GAT AAA TTG GAT GTG GTT GAT      3024
Arg Asp Gly Phe Ala Glu Leu Gln Met Asp Lys Leu Asp Val Val Asp
        995                 1000                1005

AGT TTT GGA ACT GTT CCC TTC CTT GAC TAC AAA CAT TTT GCT CTG AGA      3072
```

```
Ser Phe Gly Thr Val Pro Phe Leu Asp Tyr Lys His Phe Ala Leu Arg
    1010                1015                1020

ACT TTC TTC CCT GAG TCA GGT GGC TTC ACC CAC ATC TTC ACT GAA GAT      3120
Thr Phe Phe Pro Glu Ser Gly Gly Phe Thr His Ile Phe Thr Glu Asp
1025                1030                1035                1040

ATG CAT AAC AGA GAC GCC AAC GAC AAG AAT GAA AGT CTC ACA GCT TTG      3168
Met His Asn Arg Asp Ala Asn Asp Lys Asn Glu Ser Leu Thr Ala Leu
                1045                1050                1055

GAT GCC CTA ATC TGT AAT AAA AGC TTT CTT GTT ACT GTC ATC CAC ACC      3216
Asp Ala Leu Ile Cys Asn Lys Ser Phe Leu Val Thr Val Ile His Thr
            1060                1065                1070

CTT GAA AAG CAG AAG AAC TTT TCT GTG AAG GAC AGG TGT CTG TTT GCC      3264
Leu Glu Lys Gln Lys Asn Phe Ser Val Lys Asp Arg Cys Leu Phe Ala
        1075                1080                1085

TCC TTC CTA ACC ATT GCA CTG CAA ACC AAG CTG GTC TAC CTG ACC AGC      3312
Ser Phe Leu Thr Ile Ala Leu Gln Thr Lys Leu Val Tyr Leu Thr Ser
    1090                1095                1100

ATC CTA GAG GTG CTG ACC AGG GAC TTG ATG GAA CAG TGT AGT AAC ATG      3360
Ile Leu Glu Val Leu Thr Arg Asp Leu Met Glu Gln Cys Ser Asn Met
1105                1110                1115                1120

CAG CCG AAA CTC ATG CTG AGA CGC ACG GAG TCC GTC GTC GAA AAA CTC      3408
Gln Pro Lys Leu Met Leu Arg Arg Thr Glu Ser Val Val Glu Lys Leu
                1125                1130                1135

CTC ACA AAC TGG ATG TCC GTC TGC CTT TCT GGA TTT CTC CGG GAG ACT      3456
Leu Thr Asn Trp Met Ser Val Cys Leu Ser Gly Phe Leu Arg Glu Thr
            1140                1145                1150

GTC GGA GAG CCC TTC TAT TTG CTG GTG ACG ACT CTG AAC CAG AAA ATT      3504
Val Gly Glu Pro Phe Tyr Leu Leu Val Thr Thr Leu Asn Gln Lys Ile
        1155                1160                1165

AAC AAG GGT CCC GTG GAT GTA ATC ACT TGC AAA GCC CTG TAC ACA CTT      3552
Asn Lys Gly Pro Val Asp Val Ile Thr Cys Lys Ala Leu Tyr Thr Leu
    1170                1175                1180

AAT GAA GAC TGG CTG TTG TGG CAG GTT CCG GAA TTC AGT ACT GTG GCA      3600
Asn Glu Asp Trp Leu Leu Trp Gln Val Pro Glu Phe Ser Thr Val Ala
1185                1190                1195                1200

TTA AAC GTC GTC TTT GAA AAA ATC CCG GAA AAC GAG AGT GCA GAT GTC      3648
Leu Asn Val Val Phe Glu Lys Ile Pro Glu Asn Glu Ser Ala Asp Val
                1205                1210                1215

TGT CGG AAT ATT TCA GTC AAT GTT CTC GAC TGT GAC ACC ATT GGC CAA      3696
Cys Arg Asn Ile Ser Val Asn Val Leu Asp Cys Asp Thr Ile Gly Gln
            1220                1225                1230
```

```
GCC AAA GAA AAG ATT TTC CAA GCA TTC TTA AGC AAA AAT GGC TCT CCT      3744
Ala Lys Glu Lys Ile Phe Gln Ala Phe Leu Ser Lys Asn Gly Ser Pro
        1235                1240                1245

TAT GGA CTT CAG CTT AAT GAA ATT GGT CTT GAG CTT CAA ATG GGC ACA      3792
Tyr Gly Leu Gln Leu Asn Glu Ile Gly Leu Glu Leu Gln Met Gly Thr
    1250                1255                1260

CGA CAG AAA GAA CTT CTG GAC ATC GAC AGT TCC TCC GTG ATT CTT GAA      3840
Arg Gln Lys Glu Leu Leu Asp Ile Asp Ser Ser Ser Val Ile Leu Glu
1265                1270                1275                1280

GAT GGA ATC ACC AAG CTA AAC ACC ATT GGC CAC TAT GAG ATA TCA AAT      3888
Asp Gly Ile Thr Lys Leu Asn Thr Ile Gly His Tyr Glu Ile Ser Asn
                1285                1290                1295

GGA TCC ACT ATA AAA GTC TTT AAG AAG ATA GCA AAT TTT ACT TCA GAT      3936
Gly Ser Thr Ile Lys Val Phe Lys Lys Ile Ala Asn Phe Thr Ser Asp
            1300                1305                1310

GTG GAG TAC TCG GAT GAC CAC TGC CAT TTG ATT TTA CCA GAT TCG GAA      3984
Val Glu Tyr Ser Asp Asp His Cys His Leu Ile Leu Pro Asp Ser Glu
        1315                1320                1325

GCA TTC CAA GAT GTG CAA GGA AAG AGA CAT CGA GGG AAG CAC AAG TTC      4032
Ala Phe Gln Asp Val Gln Gly Lys Arg His Arg Gly Lys His Lys Phe
    1330                1335                1340

AAA GTA AAA GAA ATG TAT CTG ACA AAG CTG CTG TCG ACC AAG GTG GCA      4080
Lys Val Lys Glu Met Tyr Leu Thr Lys Leu Leu Ser Thr Lys Val Ala
1345                1350                1355                1360

ATT CAT TCT GTG CTT GAA AAA CTT TTT AGA AGC ATT TGG AGT TTA CCC      4128
Ile His Ser Val Leu Glu Lys Leu Phe Arg Ser Ile Trp Ser Leu Pro
                1365                1370                1375

AAC AGC AGA GCT CCA TTT GCT ATA AAA TAC TTT TTT GAC TTT TTG GAC      4176
Asn Ser Arg Ala Pro Phe Ala Ile Lys Tyr Phe Phe Asp Phe Leu Asp
            1380                1385                1390

GCC CAG GCT GAA AAC AAA AAA ATC ACA GAT CCT GAC GTC GTA CAT ATT      4224
Ala Gln Ala Glu Asn Lys Lys Ile Thr Asp Pro Asp Val Val His Ile
        1395                1400                1405

TGG AAA ACA AAC AGC CTT CCT CTT CGC TTC TGG GTA AAC ATC CTG AAG      4272
Trp Lys Thr Asn Ser Leu Pro Leu Arg Phe Trp Val Asn Ile Leu Lys
    1410                1415                1420

AAC CCT CAG TTT GTC TTT GAC ATT AAG AAG ACA CCA CAT ATA GAC GGC      4320
Asn Pro Gln Phe Val Phe Asp Ile Lys Lys Thr Pro His Ile Asp Gly
1425                1430                1435                1440

TGT TTG TCA GTG ATT GCC CAG GCA TTC ATG GAT GCA TTT TCT CTC ACA      4368
Cys Leu Ser Val Ile Ala Gln Ala Phe Met Asp Ala Phe Ser Leu Thr
                1445                1450                1455
```

```
GAG CAG CAA CTA GGG AAG GAA GCA CCA ACT AAT AAG CTT CTC TAT GCC      4416
Glu Gln Gln Leu Gly Lys Glu Ala Pro Thr Asn Lys Leu Leu Tyr Ala
            1460                1465                1470

AAG GAT ATC CCA ACC TAC AAA GAA GAA GTA AAA TCT TAT TAC AAA GCA      4464
Lys Asp Ile Pro Thr Tyr Lys Glu Glu Val Lys Ser Tyr Tyr Lys Ala
        1475                1480                1485

ATC AGG GAT TTG CCT CCA TTG TCA TCC TCA GAA ATG GAA GAA TTT TTA      4512
Ile Arg Asp Leu Pro Pro Leu Ser Ser Ser Glu Met Glu Glu Phe Leu
    1490                1495                1500

ACT CAG GAA TCT AAG AAA CAT GAA AAT GAA TTT AAT GAA GAA GTG GCC      4560
Thr Gln Glu Ser Lys Lys His Glu Asn Glu Phe Asn Glu Glu Val Ala
1505                1510                1515                1520

TTG ACA GAA ATT TAC AAA TAC ATC GTA AAA TAT TTT GAT GAG ATT CTA      4608
Leu Thr Glu Ile Tyr Lys Tyr Ile Val Lys Tyr Phe Asp Glu Ile Leu
                1525                1530                1535

AAT AAA CTA GAA AGA GAA CGA GGG CTG GAA GAA GCT CAG AAA CAA CTC      4656
Asn Lys Leu Glu Arg Glu Arg Gly Leu Glu Glu Ala Gln Lys Gln Leu
            1540                1545                1550

TTG CAT GTA AAA GTC TTA TTT GAT GAA AAG AAG AAA TGC AAG TGG ATG      4704
Leu His Val Lys Val Leu Phe Asp Glu Lys Lys Lys Cys Lys Trp Met
        1555                1560                1565

TAA                                                                 4707
 *
```

INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1569 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Glu Val Ser Arg Arg Lys Ala Pro Pro Arg Pro Pro Arg Pro Ala
  1               5                  10                  15

Ala Pro Leu Pro Leu Leu Ala Tyr Leu Leu Ala Leu Ala Ala Pro Gly
             20                  25                  30

Arg Gly Ala Asp Glu Pro Val Trp Arg Ser Glu Gln Ala Ile Gly Ala
         35                  40                  45

Ile Ala Ala Ser Gln Glu Asp Gly Val Phe Val Ala Ser Gly Ser Cys
```

```
      50                  55                  60

Leu Asp Gln Leu Asp Tyr Ser Leu Glu His Ser Leu Ser Arg Leu Tyr
 65                  70                  75                  80

Arg Asp Gln Ala Gly Asn Cys Thr Glu Pro Val Ser Leu Ala Pro Pro
                 85                  90                  95

Ala Arg Pro Arg Pro Gly Ser Ser Phe Ser Lys Leu Leu Leu Pro Tyr
            100                 105                 110

Arg Glu Gly Ala Ala Gly Leu Gly Gly Leu Leu Leu Thr Gly Trp Thr
        115                 120                 125

Phe Asp Arg Gly Ala Cys Glu Val Arg Pro Leu Gly Asn Leu Ser Arg
    130                 135                 140

Asn Ser Leu Arg Asn Gly Thr Glu Val Val Ser Cys His Pro Gln Gly
145                 150                 155                 160

Ser Thr Ala Gly Val Val Tyr Arg Ala Gly Arg Asn Asn Arg Trp Tyr
                165                 170                 175

Leu Ala Val Ala Ala Thr Tyr Val Leu Pro Glu Pro Glu Thr Ala Ser
            180                 185                 190

Arg Cys Asn Pro Ala Ala Ser Asp His Asp Thr Ala Ile Ala Leu Lys
        195                 200                 205

Asp Thr Glu Gly Arg Ser Leu Ala Thr Gln Glu Leu Gly Arg Leu Lys
    210                 215                 220

Leu Cys Glu Gly Ala Gly Ser Leu His Phe Val Asp Ala Phe Leu Trp
225                 230                 235                 240

Asn Gly Ser Ile Tyr Phe Pro Tyr Tyr Pro Tyr Asn Tyr Thr Ser Gly
                245                 250                 255

Ala Ala Thr Gly Trp Pro Ser Met Ala Arg Ile Ala Gln Ser Thr Glu
            260                 265                 270

Val Leu Phe Gln Gly Gln Ala Ser Leu Asp Cys Gly His Gly His Pro
        275                 280                 285

Asp Gly Arg Arg Leu Leu Leu Ser Ser Ser Leu Val Glu Ala Leu Asp
    290                 295                 300

Val Trp Ala Gly Val Phe Ser Ala Ala Ala Gly Glu Gly Gln Glu Arg
305                 310                 315                 320

Arg Ser Pro Thr Thr Thr Ala Leu Cys Leu Phe Arg Met Ser Glu Ile
                325                 330                 335

Gln Ala Arg Ala Lys Arg Val Ser Trp Asp Phe Lys Thr Ala Glu Ser
            340                 345                 350
```

```
His Cys Lys Glu Gly Asp Gln Pro Glu Arg Val Gln Pro Ile Ala Ser
        355                 360                 365

Ser Thr Leu Ile His Ser Asp Leu Thr Ser Val Tyr Gly Thr Val Val
    370                 375                 380

Met Asn Arg Thr Val Leu Phe Leu Gly Thr Gly Asp Gly Gln Leu Leu
385                 390                 395                 400

Lys Val Ile Leu Gly Glu Asn Leu Thr Ser Asn Cys Pro Glu Val Ile
                405                 410                 415

Tyr Glu Ile Lys Glu Glu Thr Pro Val Phe Tyr Lys Leu Val Pro Asp
            420                 425                 430

Pro Val Lys Asn Ile Tyr Ile Tyr Leu Thr Ala Gly Lys Glu Val Arg
        435                 440                 445

Arg Ile Arg Val Ala Asn Cys Asn Lys His Lys Ser Cys Ser Glu Cys
    450                 455                 460

Leu Thr Ala Thr Asp Pro His Cys Gly Trp Cys His Ser Leu Gln Arg
465                 470                 475                 480

Cys Thr Phe Gln Gly Asp Cys Val His Ser Glu Asn Leu Glu Asn Trp
                485                 490                 495

Leu Asp Ile Ser Ser Gly Ala Lys Lys Cys Pro Lys Ile Gln Ile Ile
            500                 505                 510

Arg Ser Ser Lys Glu Lys Thr Thr Val Thr Met Val Gly Ser Phe Ser
        515                 520                 525

Pro Arg His Ser Lys Cys Met Val Lys Asn Val Asp Ser Ser Arg Glu
    530                 535                 540

Leu Cys Gln Asn Lys Ser Gln Pro Asn Arg Thr Cys Thr Cys Ser Ile
545                 550                 555                 560

Pro Thr Arg Ala Thr Tyr Lys Asp Val Ser Val Val Asn Val Met Phe
                565                 570                 575

Ser Phe Gly Ser Trp Asn Leu Ser Asp Arg Phe Asn Phe Thr Asn Cys
            580                 585                 590

Ser Ser Leu Lys Glu Cys Pro Ala Cys Val Glu Thr Gly Cys Ala Trp
        595                 600                 605

Cys Lys Ser Ala Arg Arg Cys Ile His Pro Phe Thr Ala Cys Asp Pro
    610                 615                 620

Ser Asp Tyr Glu Arg Asn Gln Glu Gln Cys Pro Val Ala Val Glu Lys
625                 630                 635                 640
```

Thr Ser Gly Gly Gly Arg Pro Lys Glu Asn Lys Gly Asn Arg Thr Asn
645 650 655

Gln Ala Leu Gln Val Phe Tyr Ile Lys Ser Ile Glu Pro Gln Lys Val
660 665 670

Ser Thr Leu Gly Lys Ser Asn Val Ile Val Thr Gly Ala Asn Phe Thr
675 680 685

Arg Ala Ser Asn Ile Thr Met Ile Leu Lys Gly Thr Ser Thr Cys Asp
690 695 700

Lys Asp Val Ile Gln Val Ser His Val Leu Asn Asp Thr His Met Lys
705 710 715 720

Phe Ser Leu Pro Ser Ser Arg Lys Glu Met Lys Asp Val Cys Ile Gln
725 730 735

Phe Asp Gly Gly Asn Cys Ser Ser Val Gly Ser Leu Ser Tyr Ile Ala
740 745 750

Leu Pro His Cys Ser Leu Ile Phe Pro Ala Thr Thr Trp Ile Ser Gly
755 760 765

Gly Gln Asn Ile Thr Met Met Gly Arg Asn Phe Asp Val Ile Asp Asn
770 775 780

Leu Ile Ile Ser His Glu Leu Lys Gly Asn Ile Asn Val Ser Glu Tyr
785 790 795 800

Cys Val Ala Thr Tyr Cys Gly Phe Leu Ala Pro Ser Leu Lys Ser Ser
805 810 815

Lys Val Arg Thr Asn Val Thr Val Lys Leu Arg Val Gln Asp Thr Tyr
820 825 830

Leu Asp Cys Gly Thr Leu Gln Tyr Arg Glu Asp Pro Arg Phe Thr Gly
835 840 845

Tyr Arg Val Glu Ser Glu Val Asp Thr Glu Leu Glu Val Lys Ile Gln
850 855 860

Lys Glu Asn Asp Asn Phe Asn Ile Ser Lys Lys Asp Ile Glu Ile Thr
865 870 875 880

Leu Phe His Gly Glu Asn Gly Gln Leu Asn Cys Ser Phe Glu Asn Ile
885 890 895

Thr Arg Asn Gln Asp Leu Thr Thr Ile Leu Cys Lys Ile Lys Gly Ile
900 905 910

Lys Thr Ala Ser Thr Ile Ala Asn Ser Ser Lys Lys Val Arg Val Lys
915 920 925

Leu Gly Asn Leu Glu Leu Tyr Val Glu Gln Glu Ser Val Pro Ser Thr

930 935 940

Trp Tyr Phe Leu Ile Val Leu Pro Val Leu Leu Val Ile Val Ile Phe
945 950 955 960

Ala Ala Val Gly Val Thr Arg His Lys Ser Lys Glu Leu Ser Arg Lys
965 970 975

Gln Ser Gln Gln Leu Glu Leu Leu Glu Ser Glu Leu Arg Lys Glu Ile
980 985 990

Arg Asp Gly Phe Ala Glu Leu Gln Met Asp Lys Leu Asp Val Val Asp
995 1000 1005

Ser Phe Gly Thr Val Pro Phe Leu Asp Tyr Lys His Phe Ala Leu Arg
1010 1015 1020

Thr Phe Phe Pro Glu Ser Gly Gly Phe Thr His Ile Phe Thr Glu Asp
1025 1030 1035 1040

Met His Asn Arg Asp Ala Asn Asp Lys Asn Glu Ser Leu Thr Ala Leu
1045 1050 1055

Asp Ala Leu Ile Cys Asn Lys Ser Phe Leu Val Thr Val Ile His Thr
1060 1065 1070

Leu Glu Lys Gln Lys Asn Phe Ser Val Lys Asp Arg Cys Leu Phe Ala
1075 1080 1085

Ser Phe Leu Thr Ile Ala Leu Gln Thr Lys Leu Val Tyr Leu Thr Ser
1090 1095 1100

Ile Leu Glu Val Leu Thr Arg Asp Leu Met Glu Gln Cys Ser Asn Met
1105 1110 1115 1120

Gln Pro Lys Leu Met Leu Arg Arg Thr Glu Ser Val Val Glu Lys Leu
1125 1130 1135

Leu Thr Asn Trp Met Ser Val Cys Leu Ser Gly Phe Leu Arg Glu Thr
1140 1145 1150

Val Gly Glu Pro Phe Tyr Leu Leu Val Thr Thr Leu Asn Gln Lys Ile
1155 1160 1165

Asn Lys Gly Pro Val Asp Val Ile Thr Cys Lys Ala Leu Tyr Thr Leu
1170 1175 1180

Asn Glu Asp Trp Leu Leu Trp Gln Val Pro Glu Phe Ser Thr Val Ala
1185 1190 1195 1200

Leu Asn Val Val Phe Glu Lys Ile Pro Glu Asn Glu Ser Ala Asp Val
1205 1210 1215

Cys Arg Asn Ile Ser Val Asn Val Leu Asp Cys Asp Thr Ile Gly Gln
1220 1225 1230

Ala Lys Glu Lys Ile Phe Gln Ala Phe Leu Ser Lys Asn Gly Ser Pro
1235 1240 1245

Tyr Gly Leu Gln Leu Asn Glu Ile Gly Leu Glu Leu Gln Met Gly Thr
1250 1255 1260

Arg Gln Lys Glu Leu Leu Asp Ile Asp Ser Ser Ser Val Ile Leu Glu
1265 1270 1275 1280

Asp Gly Ile Thr Lys Leu Asn Thr Ile Gly His Tyr Glu Ile Ser Asn
1285 1290 1295

Gly Ser Thr Ile Lys Val Phe Lys Lys Ile Ala Asn Phe Thr Ser Asp
1300 1305 1310

Val Glu Tyr Ser Asp Asp His Cys His Leu Ile Leu Pro Asp Ser Glu
1315 1320 1325

Ala Phe Gln Asp Val Gln Gly Lys Arg His Arg Gly Lys His Lys Phe
1330 1335 1340

Lys Val Lys Glu Met Tyr Leu Thr Lys Leu Leu Ser Thr Lys Val Ala
1345 1350 1355 1360

Ile His Ser Val Leu Glu Lys Leu Phe Arg Ser Ile Trp Ser Leu Pro
1365 1370 1375

Asn Ser Arg Ala Pro Phe Ala Ile Lys Tyr Phe Phe Asp Phe Leu Asp
1380 1385 1390

Ala Gln Ala Glu Asn Lys Lys Ile Thr Asp Pro Asp Val Val His Ile
1395 1400 1405

Trp Lys Thr Asn Ser Leu Pro Leu Arg Phe Trp Val Asn Ile Leu Lys
1410 1415 1420

Asn Pro Gln Phe Val Phe Asp Ile Lys Lys Thr Pro His Ile Asp Gly
1425 1430 1435 1440

Cys Leu Ser Val Ile Ala Gln Ala Phe Met Asp Ala Phe Ser Leu Thr
1445 1450 1455

Glu Gln Gln Leu Gly Lys Glu Ala Pro Thr Asn Lys Leu Leu Tyr Ala
1460 1465 1470

Lys Asp Ile Pro Thr Tyr Lys Glu Glu Val Lys Ser Tyr Tyr Lys Ala
1475 1480 1485

Ile Arg Asp Leu Pro Pro Leu Ser Ser Ser Glu Met Glu Glu Phe Leu
1490 1495 1500

Thr Gln Glu Ser Lys Lys His Glu Asn Glu Phe Asn Glu Glu Val Ala
1505 1510 1515 1520

Leu Thr Glu Ile Tyr Lys Tyr Ile Val Lys Tyr Phe Asp Glu Ile Leu
1525 1530 1535

Asn Lys Leu Glu Arg Glu Arg Gly Leu Glu Glu Ala Gln Lys Gln Leu
1540 1545 1550

Leu His Val Lys Val Leu Phe Asp Glu Lys Lys Lys Cys Lys Trp Met
1555 1560 1565